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THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF NGF RECEPTOR GENE PRODUCTS IN SCHWANN CELLS AND FIBROBLASTS

BY

PHILIP AMOS BARKER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY AND CELL BIOLOGY

EDMONTON, ALBERTA

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PHILIP A. BARKER

Department of Anatomy and Cell Biology

University of Alberta

Edmonton, Alberta

T6G 2H7

Date: May 6, 1991

UNIVERSITY OF ALBERTA

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED CHARACTERIZATION OF NGF RECEPTOR GENE PRODUCTS IN NONNEURONAL CELLS.

SUBMITTED BY PHILIP A. BARKER IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DR. RICHARD MURPHY

DR. ANN ACHESON

DR. ROBERT CAMPENOT

DR. WARREN GALLIN

DR. GRANT MACFADDEN

DR. FREDA MILLER

DR. KENNETH NEET

DR. KERRY SIMINOSKI

DATE: MAY 2, 1991

ABSTRACT

These studies were initiated to characterize products of the nerve growth factor (NGF) receptor gene in two nonneuronal cell types, fibroblasts and Schwann cells. Our studies show that although cultured fibroblasts synthesize NGF receptor mRNA, NGF receptor protein cannot be detected within these cells when measured by equilibrium binding, ligand crosslinking and direct surface labelling followed by immunoprecipitation. Analysis of NGF receptor mRNA produced by these cells by polymerase chain reaction (PCR) and RNase protection did not reveal the presence of any splice variants. In cultured rat Schwann cells, the production of a soluble, truncated form of the NGF receptor was analyzed. Pulse chase-immunoprecipitation analysis revealed that the soluble NGF receptor is produced post-translationally from intact, surface-bound receptor. PCR and RNase protection analysis provided no evidence of alternatively spliced NGF receptor gene products which might account for the direct de novo production of this soluble receptor within Schwann cells. Further studies carried out to characterize splice variants produced by PCR revealed that this technique can produce artefactual results that, unless rigorously controlled, may produce misleading results. Together, these results show that the NGF receptor gene is differentially regulated between these two cell types, that NGF receptor mRNA produced by these cell types is identical and that the production of the truncated NGF receptor can be accounted for solely by post-translational mechanisms.

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NGF RECEPTORS: HIGH AND LOW AFFINITY

Like all protein growth factors, nerve growth factor (NGF) initiates biological activity by binding to specific receptors on the surface of cells. NGF receptors have been characterized on dorsal root ganglion (DRG) sensory and superior cervical ganglion (SCG) sympathetic neurons, on rat pheochromocytoma cells (PC12 cells, Greene and Tischler, 1976), and on human melanoma cells (A875, Fabricant et al. 1977). After binding to its receptor, NGF promotes neurite outgrowth and enhances the survival of primary sensory and sympathetic neurons in culture, and induces PC12 cells to undergo a phenotypic switch from a chromaffin-like cell to a cell with neuritic processes resembling a sympathetic neuron (Greene and Tischler, 1976). No structural or chemical changes have been detected in A875 cells treated with NGF (but see Fabricant et al. 1977).

The binding of NGF to neurons and to PC12 cells does not occur by simple first-order association. On PC12 cells, the association of NGF with its receptor at 0° C can be described by a single class of binding site with a K_d of about 2-3 nM (Herrup and Thoenen, 1979; Chandler et al. 1984; Woodruff and Neet; 1986) but at higher temperatures, NGF binding on PC12 cells is more complex. Scatchard plots of equilibrium binding of ^{125}I-NGF to the surface of PC12 cells show clear nonlinearity (Bernd and Greene, 1984; Woodruff and Neet, 1986) and bound radiolabelled NGF displaced with excess unlabelled NGF is released from primary neurons (Sutter et al. 1979; Godfrey and Shooter, 1986) and PC12 cells (Landreth and Shooter, 1980, Olender and Stach,1980; Schechter and Bothwell, 1981; Buxser et al. 1983; Bernd and Greene, 1984; Green et al. 1986; Woodruff and Neet; 1986) with two distinct dissociation components when measured at either 37° C or 0° C (Schechter and Bothwell, 1981; Sutter et al.

1979;, Tait et al.1981; Woodruff and Neet, 1986). These two binding components have been termed low affinity, fast component (ie. releases NGF fast) or Type II receptor, and the high affinity, slow component or Type I receptor, respectively. The Type I binding site forms relatively slowly; at 37 C, NGF rapidly occupies Type II sites on PC12 cells but Type I sites are detectable only after a lag of about 30 seconds (Landreth and Shooter, 1980; Kasaian and Neet, 1988). Cells that show no apparent response to NGF treatment, such as Schwann cells (Yasuda et al. 1987; DiStefano and Johnson, 1988) and A875 cells (Buxser et al. 1983a) display only Type II binding.

Several theories have been put forward to explain the relationship of the high and low affinity receptors. One explanation is that the two binding components reflect differences in the cellular location of ^{125}I-NGF rather than in the intrinsic properties of the receptor(s). According to this hypothesis, the rapidly dissociating Type II component represents surface-bound NGF and the slowly dissociating Type I component represents internalized NGF which at 37° C cycles back to the cell surface. PC12 cells treated with phenylarsine oxide, which inhibits endocytosis, fail to develop Type I NGF binding sites, and PC12 cells treated with monensin, which prevents endocytic vesicles from cycling to the cell surface, significantly increases the number of Type I binding sites (Eveleth and Bradshaw, 1988). Also, the ^{125}I-NGF bound to PC12 cells at 37° C which shows Type I binding kinetics is not measurable on the cell surface but is present in an internal compartment (Buxser et al, 1990; Kasaian and Neet, 1988). Taken together, these data suggest that development of Type I binding is a consequence of internalization of the NGF-Type II receptor complex.

An alternative hypothesis is that the Type I receptor forms on the cell

surface slowly and is then immediately internalized. To test this hypothesis directly, Kasaian and Neet (1988) compared the binding of NGF to PC12 cells at 37° C and at 4° C. At 37° C, no Type I sites were detected on the cell surface, but when binding was performed for long periods at 4° C, a temperature at which internalization will not occur, Type 1 sites did slowly form (although at a rate 5-10 times slower than occurs at 37° C). These results corroborate previous observations of Type I binding at 0° C (Schechter and Bothwell, 1981). Additional evidence for cell surface Type I binding sites comes from experiments in which a medulloblastoma cell line was transfected with cDNA encoding the Type II NGF receptor. Following transfection, abundant Type I and Type II NGF binding sites were observed even though NGF internalization was barely measurable (Pleasure et al. 1990). Together, these results suggest that type I binding sites do not arise solely as a consequence of receptor location but rather that they exist or at least may be formed on the cell surface.

Chemical crosslinking of ^{125}I-NGF to Type I and Type II receptors has been used to analyze these molecules biochemically. A variety of crosslinkers have been employed, including 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCL (EDAC), hydroxysuccinimidyl-4-azidobenzoate (HSAB), disuccinimidyl suberate (DSS), dithiobis(succinimidylpropionate) (DSP) and ethylene glycolbis(succinimidylsuccinate) (EGS). Irrespective of the crosslinker (see Table 1), on all cells that bind NGF, ^{125}I-NGF can be crosslinked into a 100-110 kD complex that consists of NGF and the Type II receptor (Johnson et al. 1986; Radeke et al. 1987). A second, 200-225 kD crosslinked species observed by several authors is likely a dimer of the Type II receptor (Buxser et al. 1983).

A second complex (140-160 kd) observed following treatment with the hydrophobic crosslinkers HSAB, DSS, DSP or EGS (but not EDAC, which is water soluble) has been proposed to contain the Type I NGF receptor (Hosang and

TABLE 1 - NGF Receptor Species Detected by Crosslinking

Receptor Source	Linker	MW	Reference
PC12 cells	EDAC	100, 225	Grob et al. 1983
PC12 cells	EDAC	103	Green and Greene, 1986
PC12 cells	EDAC	95, 220	Distefano & Johnson,1988
PC12 cells	EDAC	100	Meakin and Shooter, 1991
PC12 cells	HSAB	148	Massague et al. 1982
PC12 cells	HSAB	110, 200	Buxser et al. 1983
PC12 cells	HSAB	100, 225	Grob et al. 1983
PC12 cells	HSAB	100, 158, 225	Hosang and Shooter, 1985
PC12 cells	HSAB	103, 148	Green and Greene, 1986
PC12 cells	HSAB	100, 158	Meakin and Shooter, 1991
PC12 cells	DSS	100, 158	Meakin and Shooter, 1991
PC12 cells	EGS	100, 158	Meakin and Shooter, 1991
PC12nnr cells	EDAC	103	Green and Greene, 1986
A875 cells	EDAC	100, 225	Grob et al. 1983
A875 cells	HSAB	110, 200	Buxser et al. 1983
A875 cells	HSAB	100, 225	Grob et al. 1983
A875 cells	EDAC	90, 200	Ross et al. 1985
A875 cells	DSS	100,	Meakin and Shooter, 1991
SH-SY5Y cells	EDAC	103	Green and Greene, 1986
PCNA cells	HSAB	100	Radeke et al. 1987
PCNA cells	DSS	100	Meakin and Shooter, 1991
E1 transfectants	EDAC	100	Johnson et al. 1986
MED-NGFR	EDAC	100	Pleasure et al. 1990
RN2	DSS	100	Meakin and Shooter, 1991
Rabbit SCG	HSAB	112, 143	Massague et al. 1981
Rabbit SCG	HSAB	110, 135	Kouchalakos et al. 1986
Chick DRG neurons	HSAB	105, 145	Hosang and Shooter, 1985
Rat sympathetic neurons	EDAC	103, 200	Green and Greene, 1986
Peripheral nerve	EDAC	90, 220	Taniuchi et al. 1986
Chick DRG Schwann cells	HSAB	105	Hosang and Shooter,1985
Rat nerve Schwann cells	EDAC	95, 220	DiStefano & Johnson,1988
Rat SCG Schwann cells	EDAC	103	Green and Greene, 1986

Shooter, 1985; Greene and Greene, 1986; Meakin and Shooter, 1991). The evidence for this is that this species is only present on cells exhibiting

Type I binding and only under conditions in which only the Type I receptor is occupied. Furthermore, the Type I receptor-NGF complex but not NGF complexed with Type 2 receptors is resistant to mild trypsin treatment (Landreth and Shooter, 1980), and evidence shows that PC12 cells exposed to ^{125}I-NGF, treated with trypsin and crosslinked, retain this complex, although its mobility on SDS gels is slightly increased (to M_r 148 kd; Hosang and Shooter, 1985). If cells are first treated with trypsin then crosslinked with NGF, the 148 kd band remains detectable, suggesting that this receptor is resistant to trypsin treatment even when unoccupied (Meakin and Shooter, 1991).

Several lines of evidence suggest that the Type I receptor is made up of the Type II receptor complexed with unidentified protein(s). Scatchard analysis of NGF binding to PC12 cells at 37° C is consistent with this hypothesis (Woodruff and Neet, 1986) and analysis of ^{125}I-NGF binding by EDAC crosslinking has shown that the 100 kd complex binds NGF even under binding conditions in which virtually all binding should be to Type I sites (Green and Greene, 1986). Chymotryptic mapping of the Type I and Type II complexes identified by HSAB crosslinking of receptor within rabbit superior cervical ganglion indicated that the two may share common peptides (Massaugue et al. 1981). Furthermore, cDNA encoding the Type II NGF receptor transfected into receptor negative PC12 cells (Hempstead et al. 1989) or medulloblastoma cells (Pleasure et al. 1990) that are not responsive to NGF generates both Type I and II binding as well as NGF responsiveness, as measured by NGF-induced increases in fos mRNA. In neuroblastoma cells, transfection with the NGF receptor cDNA results in NGF-induced neurite outgrowth (Matsushima and Bogenmann, 1990). Similar transfections using cDNA lacking coding regions for most of the cytoplasmic domain of the receptor (described below) generate only

Type II binding sites; in these cells, NGF treatment had no effect on fos levels. Therefore, the intracellular domain of the Type II NGF receptor appears to be required for Type I binding (Hempstead et al. 1990).

These data suggest strongly that the Type II receptor is required for formation of the Type I site, but evidence proving that these two receptor populations are chemically related is lacking. Recent data indicates that these receptors may, in fact, be distinct molecular entities. The presumptive high affinity crosslinked complex (158 kd) can be immunoprecipitated using antibodies against NGF but not by three separate antibodies directed against the rat Type II NGF receptor, even though these same antibodies effectively immunoprecipitate the 100 kd crosslinked Type II NGF receptor. Furthermore, antibodies against phosphotyrosine immunoprecipitate Type I but not Type II receptors even under conditions in which both Type I and II sites are occupied (Meakin and Shooter, 1991). These results raise the possibility that the Type I complex may not contain the Type II NGF receptor protein, even though the Type II receptor must be present within the cell to allow Type I sites to form. The molecular nature of the Type I receptor continues to come under intense scrutiny in a number of labs and it is likely that the structure of the Type I receptor will soon become apparent.

CHARACTERIZATION OF THE TYPE II RECEPTOR

Monoclonal antibodies have been extremely useful in characterizing the NGF receptor. Ross et al. (1984) identified two NGF receptor-specific monoclonal antibodies (ME20.4 and ME82-11; raised against melanoma cells) that inhibited NGF binding, immunoprecipitated EDAC-crosslinked ^{125}I-NGF/NGF receptor complexes of 90 and 200 kD, and reacted with a 75 kD complex from

cells metabolically labelled with ^{35}S-cysteine. The ME20.4 antibody (which does not react with the rat receptor) has become widely used for analyzing NGF receptor expression in humans and other primates. A second series of monoclonal antibodies was generated against purified human NGF receptor (Marano et al. 1987). These monoclonals, designated NGFR 1-5, also inhibit binding of ^{125}I-NGF to intact melanoma cells and immunoprecipitate the NGF receptor. NGFR 4 and 5 also recognize reduced, denatured NGF receptor and have been used for receptor detection on western blots; ME20.4 detects denatured, but not reduced receptor (Vissavajjhala and Ross, 1990).

Another monoclonal antibody, MC192, specific for the rat form of the NGF receptor has also become widely used. Generated against isolated PC12 cell membranes, MC192 was isolated on the basis of its ability to increase ^{125}I-NGF binding to PC12 cells (Chandler et al. 1984). This response is mediated exclusively through the Type II receptor; both Type I binding and levels of the 158 kd HSAB-crosslinked complex are reduced following exposure to MC192 (Chandler et al. 1984). MC192 immunoprecipitates crosslinked ^{125}I-NGF/NGF receptor complexes of 90 and 200 kD (Taniuchi et al. 1986a) and immunoprecipitates a 75 kD complex from cells metabolically labelled with ^{35}S-cysteine (Radeke et al. 1986). A second monoclonal, 217c, was generated against a surface antigen from rat glia which has recently been identified as the Type II NGF receptor (Kumar et al. 1990).

Monoclonal antibodies raised against the NGF receptor permitted its cloning from human and rat. PC12 cell genomic DNA was transferred to mouse fibroblasts and a cell (PCNA) that subsequently expressed abundant NGF receptor on its surface was identified by FACS analysis using MC192. The NGF receptor cDNA was obtained by screening a complete PCNA cDNA library with

labelled PCNA cDNA subtracted against the parent nontransfected cell line. The resultant cDNA was transfected into mouse cells and shown to encode a protein that bound both NGF and MC192 (Radeke et al. 1987). Human NGF receptor was cloned by similar methods and identified by its ability to bind NGF and ME20.4 (Chao et al. 1986; Johnson et al. 1986). cDNAs for human NGF receptor predicted an amino terminal sequence virtually identical to that determined by direct amino acid sequencing of NGF receptor isolated from A875 cells (Marano et al. 1986). Recently, NGF receptor from chick has also been cloned (Large et al. 1989; Heuer et al. 1990). NGF receptor mRNA from several species has been sized and in human is 3.8 kb, from rat 3.7 kb, and from chick 4.5 kb. These differences are due to differences within the 3' noncoding region of these molecules.

The rat NGF receptor cDNA codes for a protein of 427 amino acids (predicted M_r 45.5) which includes a 29 amino acid signal peptide; once cleaved, the mature protein has predicted mass of 42.5 kD. A single putative transmembrane domain of 22 amino acids separates an extracellular domain of 222 amino acids and an intracellular domain of 151 amino acids. The extracellular domain contains 4 repeating cysteine-rich regions, each 35-42 amino acids long with the relative positions of the 6 cysteine residues well-conserved in each. The third and fourth cysteine rich domains are highly acidic, each containing 18 negatively charged residues. Two potential sites for N-linked glycosylation (Asn-X-Ser/Thr) were identified within the extracellular domain of the rat cDNA; however, one was likely due to a cloning artifact (Dr. Susan Meakin, personal communication) leaving only a single N-linked glycosylation site which is conserved in both the human and chicken receptor sequences. The cysteine-rich and transmembrane domains are connected

by a region rich in threonine, serine and proline residues which has led to the suggestion that this domain is a likely site for O-linked glycosylation. The intracellular domain of the NGF receptor has no homology to any known protein and contains few identifiable structural motifs other than a PEST sequence rich in proline, aspartate, glutamate, serine and threonine residues; similar regions have been implicated in targeting proteins for rapid degradation (Rogers et al. 1986; Recksteiner et al. 1987). Recently, the predicted tertiary structure of a number of receptors have been compared in an attempt to discern related structural motifs (Feinstein and Larhammer, 1990). This analysis revealed that NGF receptor and a number of other receptors have a domain resembling that in mastoparan, a peptide which has stimulatory effects on several GTPases. The functional significance of this finding remains to be determined but it raises the possibility that NGF receptor may interact with a G protein as part of its signal transduction cascade.

The amino acid sequences of the rat and human receptors are virtually identical. Because the chick sequence is more divergent, specific domains which are particularly well conserved between these species can be identified (Large et al. 1989 - see Figure 1). The most strongly conserved domain contains the sequence stretching from the last 19 residues of the extracellular region to the 46th residue of the cytoplasmic region. This portion is 95% identical in the NGF receptors of human, rat and chick and is perfectly conserved across the transmembrane domains of all three species, suggesting that this region plays an important functional role. The finding that the transmembrane domain is absolutely conserved is unusual and implies that this region may mediate the interaction of the NGF receptor with other highly conserved membrane-associated molecules. The COOH terminus of the

molecule is also highly conserved. Eighty one percent of the residues in this region are identical and the remainder are conservative substitutions. It will be important to compare the structure of NGF receptors from still more evolutionarily divergent species.

The cysteine-rich extracellular domains of the NGF receptor are homologous to extracellular domains within a number of distinct cell surface proteins and to a secreted protein, T2, which acts as a soluble form of the TNF-α and TNF-β (Smith et al. in press). Although the homology extends to a variety of residues, the most obvious similarity between the molecules listed in Table 2 is the conservation of cysteine residue positions. Also, with the exception of T2, all of these molecules contain an extracellular membrane-proximal domain rich in serine, threonine and proline suggesting that they all may share some common structural motif.

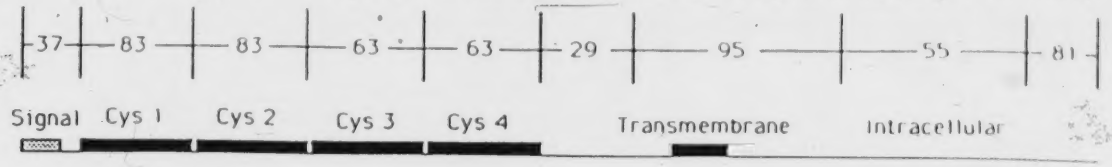
Table 2 - Molecules Showing Sequence Similarity to NGF Receptor

TNFR	Specific receptor for TNF (Smith et al. 1990)
T2	Secreted shope fibroma viral protein (Upton et al. 1987)
CD40	B cell specific surface antigen (Stamenkovic et al. 1989)
mu4-1BB	T cell specific surface antigen (Kwon and Weissman, 1989)
MRC OX40	CD4+ T cell surface antigen (Mallett et al 1990)

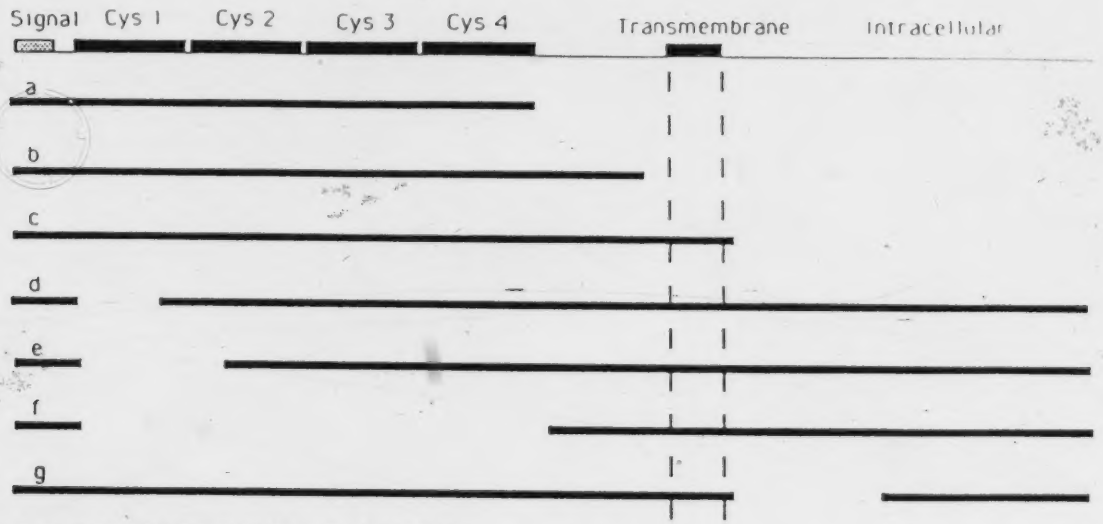
Analysis of cDNA deletion mutants have generated some structure/function information about the receptor (Figure 1). Deletion of most of the cytoplasmic domain results in loss of Type I binding, functional responses to NGF and the ability to crosslink ^{125}I-NGF into a 150 kD complex using HSAB (Hempstead et al. 1990). The deletion does not disrupt Type II binding (Hempstead et al. 1990) or binding of the ME20.4, ME82-11, NGFR5 or MC192 monoclonal antibodies (Reddy et al. 1990; Welcher et al.1991). Rat NGF receptor with deletions in the first or in the first and second cysteine-rich extracellular domains

Figure 1: Structural Analysis of NGF Receptor

1) Percent homology between regions of chick and rat/human NGF receptor (from Large et al. 1989)



2) NGF Receptor Deletion Mutants



a, c, d, and f from Welcher et al. (1991)
b and c from Reddy et al. (1990)
c from Vissavajjhala and Ross (1990)
b, c and g from Hempstead et al. (1990)

(Figure 1, lines D and E) are not recognized by MC192 but still bind NGF, albeit with reduced affinity. NGF receptor in which all four cysteine-rich domains are deleted binds neither NGF or MC192 (Welcher et al. 1991). A secreted truncated receptor has been identified (see below) that apparently lacks transmembrane and intracellular domains but which binds NGF. Truncated forms produced by cDNA deletions which contain the 4 cysteine rich repeats but lack the membrane-proximal serine/threonine rich domain also bind both NGF and species-specific monoclonal antibodies (Reddy et al. 1990; Welcher et al. 1991). Thus, the cysteine-rich domains of the receptor appear to be sufficient for formation of the NGF binding pocket, but the contribution of each of these is still unknown.

A number of approaches have provided information on post-translational modifications of the NGF receptor. The presence of N-linked carbohydrate within the receptor was suggested by its strong affinity for wheat germ agglutinin (Grob et al. 1983; Marano et al. 1987) and confirmed by use of tunicamycin, an antibiotic which inhibits cellular production of N-linked carbohydrate. Receptors generated in cells cultured in the presence of tunicamycin which were crosslinked to ^{125}I-NGF migrated more rapidly on SDS-PAGE than receptor from control cells (Grob et al. 1983) and pulse chase analysis of receptor from tunicamycin-treated cells has an apparent molecular weight that is about 5 kd lower than that found in untreated cells (Grob et al. 1985). Pulse chase analysis has also shown that receptors synthesized in the presence of tunicamycin undergoes additional forms of post-translational modifications, probably including O-linked glycosylation and phosphorylation. Although O-linked carbohydrate has not been identified on the intact receptor, a truncated form of the NGF receptor isolated from human urine does contain

this modification (Zupan et al. 1989).

How glycosylation contributes to receptor function is not clear. N-linked sugar on the receptor is probably not required for NGF's actions (Baribault and Neet,1985) since NGF can bind to and elicit differentiation of PC12 cells grown in the presence of tunicamycin. Truncated NGF receptor produced in baculovirus lacks both N- and O-linked carbohydrate but still binds NGF and ME20.4 (Vissavajjhala and Ross, 1990). Interestingly, WGA bound to NGF responsive cells converts Type II receptors on PC12 cells to receptors which, on the basis of their slow dissociation rate and Triton X100 insolubility, appear similar to the Type I receptor (Vale and Shooter, 1982). Their formation is unlikely due to WGA-induced internalization since they are formed both at 4° and 37° C as well as in the presence of metabolic inhibitors (Vale and Shooter, 1982; Grob and Bothwell, 1983). However, these are unlikely to be normal Type I binding sites since WGA treatment of A875 cells, which are NGF unresponsive and normally unable to form Type I sites, gives results identical to those obtained with PC12 cells (Grob and Bothwell, 1983, Buxser et al. 1983).

Other post-translational modifications of the NGF receptor have been described. The extracellular domain of the protein contains intrachain disulphide bonds (Massague et al.1981; Grob et al. 1983; Vissavajjhala and Ross, 1990) and the intracellular domain is phosphorylated on serine and threonine but not on tyrosine residues (Grob et al. 1985). Treatment with NGF does not affect the phosphorylation state of the NGF receptor within A875 cells (Grob et al. 1985); the phosphorylation state of the Type II receptor within cells that display Type I binding has not been determined. As mentioned above, the 158 kD complex identified as the high affinity receptor contains

phosphotyrosine but the Type II complex, which contains bound NGF, is not immunoprecipitated by phosphotyrosine antibodies (Meakin and Shooter, 1991). Because the identification of this complex depends upon binding of ^{125}I-NGF, it is not yet known if tyrosine phosphorylation of the Type I receptor is NGF-dependent.

TYPE II RECEPTOR EXPRESSION IN VIVO

NGF receptor expression in vivo has been measured by a variety of means. Specific binding of ^{125}I-NGF to tissue sections is a valuable technique and has been used to study the expression of NGF binding sites during development and following neuronal injury (Raivich et al. 1985; Raivich et al. 1987 ; Richardson et al. 1986, Riopelle et al. 1987, Raivich and Kreutzberg, 1987, Verge et al. 1989). Advantages of this method include 1) that NGF binding sites are measured directly, 2) binding specificity can be readily established, and 3) Type I NGF binding sites can be identified. Receptor specific monoclonal antibodies have also been widely used to monitor the expression of the NGF receptor (Yan and Johnson, 1988; Schatteman et al, 1988; Schatteman et al. 1990; Yip and Johnson, 1987). However, though these antibodies are believed to be specific for the NGF receptor, the possibility that they may cross react with other molecules cannot be ruled out (see for example Peacocke et al. 1988). In a limited number of studies, immunostaining results produced using receptor antibodies have been confirmed by crosslinking/ immunoprecipitation methods or by Western blotting (eg. Taniuchi et al. 1985) but this complete analysis is seldom performed. Another technique useful for monitoring NGF receptor expression is in situ hybridization using the cloned type II NGF receptor cDNA as probe (Heuer et al. 1990, Ernfors et

al. 1988; Koh et al. 1989, Hallbrook et al. 1990). These techniques are very useful in assessing mRNA levels within individual tissues or cells; in assessing these results, however, it is important to remember that the level of a specific mRNA may not necessarily correlate with the expression of its protein product. Another useful technique for identifying NGF receptors in vivo is the retrograde transport of either ^{125}I-NGF (Hendry et al. 1984; Richardson and Riopelle, 1984) or of monoclonal antibodies directed against NGF receptor (Johnson et al. 1987). Although this provides a means of identifying specific NGF binding sites on neurons in vivo, Yan et al (1988) have noted that this transport may occur through both Type I and II NGF receptors. This is not surprising since a wide variety of molecules which bind to neuronal membranes are retrogradely transported.

Given these caveats, it is reassuring to find that, in general, results from these different types of analyses agree reasonably well. Taken in toto, several important points emerge. Most importantly, NGF receptors are detectable within neurons previously shown to be NGF dependent in vitro and in vivo. Sympathetic and neural crest derived sensory neurons both have NGF receptors by each of the techniques outlined above. Cholinergic neurons within the adult central nervous system have similarly been identified. However, it should also be noted that during development and in the adult, a wide variety of neural tissues, some not known to be NGF responsive, also appear to bear NGF receptors, for reasons unknown (Raivich et al. 1985, 1987; Ernfors et al. 1988, Yan and Johnson, 1988, Schatteman et al, 1988, Heuer et al. 1988; Hallbrook et al. 1990; Altar et al. 1991).

NGF receptor expression is detectable early in development. In situ hybridization studies revealed NGF receptor mRNA within E3 chick neural tube

and both ^{125}I-NGF binding and mRNA are observed within presumptive postmitotic neuroblasts in the mantle layer of the chick CNS beginning at E4 (Heuer et al. 1990). At later times, NGF binding and receptor mRNA are detectable at various stages in virtually all neural crest- and placodal-derived sensory ganglia, in retinal ganglion cells, in parasympathetic ganglia, throughout the medulla and pons, and within brainstem motor nuclei (Raivich et al. 1987; Heuer et al. 1990; Altar et al. 1991). The highest levels of mRNA expression occur within E8-E10 motoneurons within the lateral columns of the chick spinal cord; this level of expression exceeds those observed within all other neuronal structures at any stage of development (Ernfors et al. 1988; Ernfors et al. 1989; Heuer et al. 1990). Binding and mRNA expression has also been observed within the cerebellar anlage and later, on Purkinje cells and cells within the external granular layer (Pioro and Cuello, 1988; Eckenstein, 1988; Cohen-Cory et al. 1988; Wanaka and Johnson, 1990). A large number of other regions of the adult rat CNS have recently been shown to contain both Type I and II binding sites (Altar et al. 1991). The role of the receptor in these diverse CNS structures remains unknown.

In addition to neuronal expression, NGF receptor has also been detected in a variety of non-neuronal tissues. NGF binding, immunocytochemistry and <u>in situ</u> hybridization has shown that cells of the myotome, fusing myoblasts and Schwann cells express abundant Type II NGF receptors (Raivich et al. 1985; Taniuchi et al. 1986b; Ernfors et al 1988; Chesa et al. 1988; Heumann et al. 1989; Heuer et al. 1990). NGF receptor expression has also been observed in a number of other locations but the presence of NGF binding sites within these tissues has not been confirmed <u>in vivo</u>. These tissues include neural crest- and placode-derived cells prior to and during migration, somites prior to

differentiation, thymus, cells of the optic tract, teeth, testes, and epithelial cells surrounding hair follicles (Raivich et al. 1985, 1987; Ernfors et al. 1989; Yan and Johnson, 1988, Schatteman et al. 1988, Heuer et al. 1988; Persson et al. 1990; Byers et al. 1990). Within mesenchymal tissue, expression has been observed within developing dermatome and sclerotome, in the branchial arch, within a number of subepithelial regions and adjacent to developing hair or feather follicles (Heuer et al. 1990; Thompson et al. 1990; Byers et al.1990; Wyatt et al. 1990; Chesa et al. 1988). Although Raivich et al. (1985) detected ^{125}I-NGF binding within myotomes, no binding was observed within either sclerotome or dermatome even though these tissues express NGF mRNA (Heuer et al. 1990). Similarly, the optic tract does not bind ^{125}I-NGF (Raivich et al. 1987) although it is clearly MC192 immunoreactive (Yan and Johnson, 1988). Since both Type I and II binding sites should be detected using these ^{125}I-NGF binding techniques, these results raise the possibility that in some cases, presence of NGF receptor mRNA or MC192 reactive sites does not always correlate with ^{125}I-NGF binding.

The role that NGF receptor may be playing in these diverse non-neuronal tissues remains unknown. Taniuchi et al. (1986b) have suggested that low affinity sites present on Schwann cells may act in a haptotactic manner, presenting NGF to neurons which require it for growth and survival. Others have suggested that NGF may have roles additional to its neurotrophic function. For example, NGF has been shown to stimulate proliferation and differentiation of hematopoietic stem cells (Matsuda et al. 1988; Otten et al. 1989) and to bring about mast cell degranulation (Mazurek et al. 1986). NGF has been suggested to play a role in spermatocyte development (Ayer-LeLeive et al. 1988) and to enhance myocyte growth (Brodie and Sampson, 1987). Data have

also been presented suggesting that NGF may act as a mitogen for premitotic neuroblasts within the CNS (Cattaneo and McKay, 1990). The receptor may also play a role in responding to ligands other than NGF. NGF is a member of a family of related molecules termed neurotrophins, one of which, brain-derived neurotrophic factor (BDNF - Barde et al. 1982; Leibrock et al. 1989), binds to the Type II NGF receptor as efficiently as NGF (Rodriguez-Tabar et al. 1990). It is possible that a third closely related member of this family, neurotrophin-3 (NT-3 - Hohn et al. 1990, Rosenthal et al. 1990; Yancoupolous et al. 1990) also binds the Type II receptor. Perhaps all neurotrophins initially bind cells via the Type II receptor and only then are capable of interacting with molecules that form Type I-like binding structures. The ability of a neurotrophin to act on a cell type might require not only that the cell bear the Type II receptor but also that it contain a second molecule which forms a Type I complex specifically with that neurotrophin. This hypothesis is consistent with the finding that BDNF can completely compete NGF from the Type II receptor but only competes NGF from Type I sites when present in huge excess (Rodriguez-Tabar et al. 1990). In any event, the simple presence of the NGF receptor cannot be taken as evidence that a particular tissue or cell type is NGF responsive.

REGULATION OF NGF RECEPTOR EXPRESSION

Although it has been clear for some time that expression of the NGF receptor gene occurs in a closely regulated spatial and temporal fashion, the intrinsic or extrinsic cues that contribute to this regulation are only now beginning to be characterized. *In vivo* and *in vitro* studies have revealed that NGF treatment upregulates levels of NGF receptor mRNA within responsive cells.

NGF increases numbers of both Type I and Type II binding sites on PC12 cells (Bernd and Greene, 1984) and steady state levels of NGF receptor mRNA increase within PC12 cells (Doherty et al. 1988), sensory neurons (Lindsay et al. 1990) and sympathetic neurons (Ma et al. 1991) treated with NGF. In vivo administration of NGF results in increased NGF receptor mRNA levels in cholinergic neurons of the CNS (Higgins et al. 1989) and in peripheral sensory and sympathetic neurons (Miller et al. 1991). In PC12 cells, NGF-induced increases in NGF receptor mRNA levels are mediated at least partially at the level of transcription (Miller et al. 1991). One system in which this type of regulation may occur in vivo is within neurons of the trigeminal ganglion. Levels of NGF receptor mRNA increase shortly after the terminals of trigeminal neurons establish contact with their NGF-producing target (Davies et al. 1987, Wyatt et al ,1990). The functional significance of this increase is unknown but it is possible that neurons that initially innervate a target and receive NGF early are able to increase their receptor levels sufficiently to out-compete latecomers for a limited supply of trophic factor.

Direct tissue injury can also alter NGF receptor gene expression. Following sciatic nerve lesion, Schwann cells (Taniuchi et al. 1986; Heumann et al. 1987) and perineurial fibroblasts (Toma et al. 1991) distal to the cut rapidly express high levels of both NGF receptor mRNA and protein. Damaged motoneurons also undergo a sharp increase in NGF receptor gene expression following axotomy (Ernfors et al. 1989; Saika et al. 1991) and NGF receptor mRNA is increased within spinal cord following contusion injury (Brunello et al.1990). The factors responsible for this induction and the functional significance of these increases are unknown. Although the effect of injury upon NGF mRNA levels within peripheral neurons is not known, it is interesting

to note that axotomized sensory neurons show a decrease in their number of Type I binding sites (Verge et al. 1989).

Several other regulators of NGF receptor gene expression have been described. These include basic fibroblast growth factor (bFGF), which increases levels of NGF receptor in PC12 cells (Doherty et al. 1988) and MAH cells (a putative sympathoadrenal progenitor cell line) (Birren et al. 1990) and dexamethasone, which decreases Type II NGF binding on PC12 cells (Tocco et al. 1988) and NGF receptor mRNA in MAH cells (Birrer et al. 1990). Testosterone decreases NGF receptor mRNA levels within Sertoli cells (Persson et al. 1990) and forskolin and cyclic AMP analogues decrease cell surface NGF receptor and mRNA levels in cultured Schwann cells (Mokuno et al. 1988).

The specific trans acting factors acting upon the NGF receptor gene promoter and enhancers remain a mystery. Like several other growth factor receptor genes, the NGF receptor promoter contains no TATA box and is GC-rich (Sehgal et al. 1988). The promoter contains a perfect consensus binding site (Christy et al. 1989) for the transcriptional factor Zif-268 (aka Egr-1, Krox 24, and NGFIA; Christy et al. 1988) as well as a near-consensus steroid response element (Berg 1988); both have been suggested as likely binding sites of regulatory factors (Persson et al. 1990; Miller et al. 1991). The promoter and enhancer elements that may be involved in regulating the spatial and temporal pattern of expression or the gene's response to NGF remain unknown but transgenic mice expressing β-galactosidase under the control of 8 kb of sequence upstream of the NGF receptor transcriptional start site appear to effectively mimic both the developmental expression and injury response of the endogenous gene (Patil et al. 1990).

THE TRUNCATED NGF RECEPTOR

NGF receptor also exists in a soluble form that apparently contains the extracellular domain without the transmembrane or intracellular domains. This truncated receptor was originally identified in cell conditioned media by crosslinking methods using ^{125}I-NGF as a ligand, followed by immunoprecipitation with NGF receptor-specific monoclonal antibodies. Using this procedure, the truncated receptor was identified in media conditioned by primary Schwann cells, Schwannoma cells, PC12 cells, sympathetic neurons from superior cervical ganglion and A875 cells (DiStefano and Johnson, 1988; Zupan et al. 1989). The truncated NGF receptor is also present within urine, plasma and amniotic fluid of humans and rats (DiStefano and Johnson, 1988; Zupan et al. 1989). Levels of the protein are very high in neonates and decline with increasing age to low but detectable levels in adults; the amounts of truncated receptor detectable within urine from adult animals increase following sciatic nerve lesion and during pregnancy (DiStefano and Johnson, 1988; DiStefano et al. 1991). The factor(s) regulating production of the truncated NGF receptor have not been identified but levels of intact and truncated forms of the NGF receptor produced by Schwann cells are both decreased by axonal components (DiStefano and Chelsea, 1990).

The truncated NGF receptor within conditioned media of Schwann cells and PC12 cells has an apparent molecular weight of 65 kD when crosslinked to ^{125}I-NGF. Assuming only a single NGF molecule is present within the complex, native truncated receptor must then have an apparent molecular weight of about 50 kD. Zupan et al. (1989) have characterized the human form of the truncated NGF receptor in some detail. Sequence analyses revealed that the truncated receptor has an amino terminus identical to that of the intact receptor. Since

this protein binds both ^{125}I-NGF and NGF receptor-specific monoclonal antibodies, these data strongly suggest that the truncated and surface bound receptors are products of the same gene.

Truncated receptors can be produced by two distinct mechanisms; they can arise either by post-translational proteolysis of the intact receptor or by alternative splicing of the receptor pre-mRNA. The mechanism which generates the truncated form of the NGF receptor has not been determined. Efforts to identify specific proteolytic inhibitors which reduce production of the truncated form of the receptor have been unsuccessful (Zupan et al. 1990) and although S1 nuclease analyses have suggested that only a single NGF receptor mRNA is produced, the portion of the mRNA that encodes the transmembrane region has not been examined. If alternative splicing does account for production of truncated receptor, it is likely the transmembrane portion of the molecule that is removed by splicing to generate mRNA coding for the soluble protein. Until this region is examined specifically, the possibility that splice variants encode a truncated NGF receptor cannot be ruled out.

The production of truncated, soluble receptors is a relatively common phenomenon. Table 3 lists receptors for which truncated forms have been identified and indicates the mechanism(s) by which they are generated. Post-translational proteolysis of several intact membrane receptors has been demonstrated but the responsible proteases have not been identified. Several truncated receptors also arise from alternative splicing. In some cases, the splicing event removes the exon encoding the transmembrane domain (eg. in the Il-7 and GM-CSF receptors) and in others, an exon insertion shifts the reading frame such that the sequence encoding the transmembrane domain is out of frame and therefore not translated (eg. in the IL-4 receptor). With one exception,

TABLE 3 - A Variety of Soluble Receptors Have Been Identified

Receptor For	R_T Generated by	Reference
Il-1	Proteolysis	Symons and Duff, 1990
Il-2	Proteolysis	Loughnan et al. 1988
IgA	Proteolysis	Mostov and Bloebel, 1979
Il-4	RNA splicing	Mosley et al. 1989
Il-7	RNA splicing	Goodwin et al. 1990
EGF	RNA splicing	Petch et al, 1990
GM-CSF	RNA splicing	Ashworth and Kraft, 1990
LH-hCG	RNA splicing	Loosfelt et al. 1989
Prolactin	RNA Splicing	Zhang et al. 1990
Avian erbB	RNA Splicing	Maihle et al. 1991
TNF	Unknown	Engelmann et al. 1990
IL-6	Unknown	Novick et al. 1989
IFN-gamma	Unknown	Novick et al. 1989
Transferrin	Unknown	Shih et al. 1990
Growth Hormone	Unknown	Leung et al. 1987
IGF-II/M-6-P	Unknown	MacDonald et al. 1989

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all of the soluble receptors are derivative of receptors containing only a single transmembrane domain. The membrane-bound LH-hCG receptor is a G-protein coupled receptor and contains 7 transmembrane helices. Three separate splice variants transcribed from the LH-hCG gene have been identified and all lack sequences encoding the transmembrane domains and each is spliced onto unique 3' exons.

Despite the prevalence of truncated receptors, almost nothing is known about their physiological roles, including that of the truncated NGF receptor. Since not only NGF but also BDNF (Rodriguez-Tabar et al. 1990) and possibly NT-3 may bind the truncated NGF receptor, its biological importance may be considerable. Several functions are possible: 1) Because the truncated receptor binds ligand with avidity equal to that of the membrane form of the receptor, it can compete with the membrane receptor for ligand and may inhibit NGF's biological activity. This has been shown for truncated Il-4 receptor, which neutralizes the growth promoting activity of IL-4 *in vitro* (Mosley et

al. 1989), for truncated IL-1 receptor, which attenuates the activity of Il-1 in vivo and for recombinant β-PDGF receptor, which blocks the mitogenic effect of BB-PDGF on cultured fibroblasts (Duan et al. 1991). 2) Another role for these molecules may be to act as carrier proteins which affect the transport of ligands from the cellular source or their rates of proteolysis or clearance. This could be an important role in the action of neurotrophins since NGF is notorious for its ability to adhere nonspecifically to surfaces. Conversely, these molecules may interact with extracellular matrix and thus act to concentrate ligand. 3) Truncated receptor may act to prevent formation of active transductory complexes on the cell surface by preventing heterodimer formation of active receptor complexes. In the case of the NGF, truncated receptor might interfere with interaction of the cell surface Type II receptor with other molecule(s) required for signal transduction. This potential role is supported by data showing that a soluble form of the EGF receptor inhibits tyrosine kinase activity of the intact receptor by interfering with the formation of cell surface receptor heterodimers (Basu et al. 1989). 4) A fourth role for these molecules may be to extend the action of the respective ligand to cells which do not even contain the receptor on the cell surface. This exciting possibility is suggested by recent work with the Il-6 receptor (Hibi et al. 1990a; Hibi et al. 1990b). The Il-6 receptor shows only low affinity binding when expressed on nonresponsive cells but shows both high and low affinity binding when expressed within responsive cells. The conversion is due to a ligand-induced association of IL-6 receptor with an accessory protein (gp130) that contains the active signal transductory component of the receptor complex. Surprisingly, although transduction of the Il-6 signal requires association of gp130 with both Il-6 and the Il-6 receptor, the receptor does

not need to be membrane bound to produce this effect. Il-6 together with the soluble truncated Il-6 receptor (but not Il-6 alone) interacts with surface bound gp130 to induce a biological response. The possibility that soluble receptors may be involved in signal transduction has broad biological implications which may also be important in understanding the function of neurotrophins.

THESIS INTRODUCTION

In the following three papers, various aspects on my work on NGF receptor are presented. In the first manuscript, the mechanism by which the truncated form of the NGF receptor is generated is examined using a variety of biochemical and molecular biology techniques. My experiments show that the truncated NGF receptor derived from Schwann cells arises solely by post-translational mechanisms. The second paper is of a technical nature and describes my discovery and characterization of an "artefactual" splice variant generated by PCR. I found that when examined using standard techniques, this cDNA product appeared to be genuine and, in the absence of appropriate controls, could give very misleading results. In the third manuscript, I describe the characterization of NGF receptor gene products in dermal fibroblasts. The data indicate that, even though NGF receptor mRNA indistinguishable from that found in PC12 cells is clearly present within this cell type, a wide variety of techniques provide no evidence of surface bound NGF receptors.

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GENERATION OF THE TRUNCATED NGF RECEPTOR BY RAT SCHWANN CELLS: EVIDENCE FOR POST-TRANSLATIONAL PROCESSING.[1]

Nerve growth factor (NGF) is required for the growth, differentiation and survival of sympathetic and sensory neurons (Thoenen and Barde, 1980) and for the maintenance of cholinergic neurons of the basal forebrain (Gage et al. 1989). NGF binding to sensory and sympathetic neurons and rat pheochromocytoma cells (PC12 cells) has been well characterized and consists of a high-affinity, slow dissociating component ($Kd=10^{-11}$) and a low-affinity, fast dissociating component ($Kd=10^{-9}$)(Sutter et al. 1979; Bernd and Greene, 1984). The low affinity NGF receptor has been cloned from rat (Radeke et al. 1986), human (Johnson et al. 1986) and chick (Large et al. 1989). Receptor cDNA expressed in mouse fibroblasts generates only low affinity binding (Radeke et al. 1986; Johnson et al. 1986) but in NGF receptor-negative PC12 (Hempstead et al. 1989), medulloblastoma (Pleasure et al. 1989) or neuroblastoma (Matsushima and Bogenman, 1990) cells, expression of the receptor results in both high and low affinity binding, suggesting that high affinity binding may arise by association of the low affinity receptor with some as yet unidentified molecule. The extracellular portion of the low affinity receptor contains four repeating cysteine-rich segments that are contiguous with a serine, threonine and proline-rich sequence proximal to the membrane. The transmembrane domain and flanking sequences are well conserved between species and the intracellular domain is phosphorylated on serine and threonine residues (Grob et al. 1985). On SDS-PAGE, the receptor migrates with an apparent molecular mass of 85 kd (Johnson et al. 1986; Grob et al. 1985).

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1. A version of this chapter has been submitted to the Journal of Biological Chemistry.

A truncated form of the NGF receptor has also been described. This molecule was originally detected as a soluble NGF binding protein in media conditioned by PC12 cells and rat Schwann cells (DiStefano and Johnson, 1988). Crosslinked to NGF, the truncated NGF receptor has an apparent molecular mass of about 65 kd on SDS-PAGE. Three distinct forms of truncated NGF receptor (M_r of 45, 40 and 35 kD) have been also been detected in media conditioned by human A875 cells and within human urine (Zupan et al. 1989). The largest of these has an amino terminus identical to the extracellular portion of the intact receptor. Levels of truncated NGF receptor are high in serum, amniotic fluid and in the urine of neonates, but are barely detectable in the adult (DiStefano and Johnson, 1988; Zupan et al. 1989). The physiological role of the truncated receptor and its source remain unknown.

Truncated forms of other membrane-bound receptors have been described and are generated by one of two mechanisms. One class, which includes the Il-2 receptor (Loughlan et al. 1988) and the polymeric immunoglobulin receptor (Mostov and Simester, 1985), is generated by post-translational proteolysis of the intact, native receptor protein. Other truncated receptors, including those for Il-4 (Mosley et al. 1989), Il-6 (Novick et al. 1989), Il-7 (Goodwin et al. 1990), GM-CSF (Ashworth and Kraft, 1990) and hCH-LH (Loosfelt et al. 1989), are synthesized directly as soluble proteins as a consequence of alternative mRNA splicing.

The purpose of the studies reported here was to determine the mechanism by which the truncated form of the NGF receptor arises in rat Schwann cells. Our results indicate that the Schwann cell-derived truncated NGF receptor is not synthesized from an alternatively spliced mRNA but arises from post-translational processing of the intact receptor protein.

MATERIALS AND METHODS

Tissue Culture

Schwann cells cultures were established essentially as described (Brockes et al. 1979). Sciatic nerves from rat pups 60-72 hours old were collected aseptically and subjected to 3 x 30 minute sequential digestions with 0.25% trypsin/0.1% collagenase in serum-free Dulbecco's modified essential medium (DMEM) at 37° C. Cells were dissociated by repeated trituration through a 23G needle, washed twice in DMEM containing 10% fetal calf serum (FCS) and plated on tissue culture plastic in DMEM containing 10% FCS and 10 uM cytosine arabinoside. After three days, complement-mediated lysis was performed using anti-Thy 1.1 IgM monoclonal antibodies (NEN) to remove remaining contaminating fibroblasts. Purified Schwann cells were grown on poly-L-lysine coated tissue culture flasks in DMEM containing 10% FCS, 10 ug/ml crude glial growth factor (Gospodarowicz, 1975) and 5 ug/ml forskolin. Experiments were performed on cells between passages 5 and 10. PCNA cells, a line of L929 fibroblasts transfected with rat genomic DNA which express rat NGF receptor and pcNGFR+ cells, L929 fibroblasts transfected with the rat NGF receptor cDNA (Radeke et al. 1986)(both kindly donated by Dr. Eric Shooter, Stanford University), were maintained in high-glucose DMEM containing 10% FCS and HAT (100 uM sodium hypoxanthine, 400 nM aminopterin, 16 uM thymidine).

Antibodies

MC192, an IgG_1 monoclonal antibody specific for the rat NGF receptor (Chandler et al. 1984), was isolated from ascites fluid of mice injected with the appropriate hybridoma (kindly donated by Dr. Eugene Johnson, Washington

University) using a Pierce Immunopure kit. MOPC21, a nonspecific IgG_1 monoclonal was purchased from Organon Technika. We also used a rabbit polyclonal antibody (p3) produced against a synthetic peptide containing sequence from the intracellular domain of the chick NGF receptor (CKQNKQGANNRPVNQTPSPEGEKLHSD). This sequence is conserved in the rat in all but 2 residues (underlined). The p3 antibody was purified by affinity chromatography on columns to which the immunizing peptide was affixed.

[^{125}I]NGF Crosslinking

The 2.5S form of NGF was isolated as described (Mobley et al. 1985) with some modification (Watson et al. 1985) and radioiodinated by the lactoperoxidase method (Marchalonis, 1969) to specific activities of approximately 60 cpm/pg. For crosslinking, Schwann cells prewashed with phosphate-buffered saline were incubated with 2 nM [^{125}I]NGF in serum-free DMEM for 2 hours on ice. NGF was crosslinked to its receptor by incubation with 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for 15 minutes at room temperature. The reaction was terminated by the addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM and cells were solubilized in HEAP buffer (10 mM HEPES, 1 mM EDTA, 1 ug/ml aprotinin, 10 uM PMSF) containing 150 mM NaCl and 1% octylglucoside. ^{125}I-NGF was crosslinked to the truncated form of the NGF receptor in undiluted media which had been conditioned for 72 hours. Parallel reactions containing a 250 fold excess of unlabelled NGF were carried out in all experiments to confirm specificity.

Metabolic Labelling

Nearly confluent cultures of cells on 100 mm plates were washed twice with

prewarmed sterile phosphate buffered saline (PBS) and incubated with prewarmed cysteine-free DMEM for 20 minutes at 37° C. Prewarmed cysteine-free DMEM (2.5 ml) containing 10% dialyzed FCS and 0.4 mCi/ml [^{35}S]cysteine (1200 Ci/mmol-NEN) was added to each plate. 30 minutes later, media was removed and the cells were washed twice with prewarmed sterile PBS and incubated in fresh complete media. The chase media for Schwann cells contained 10% FCS, 10 ug/ml crude glial growth factor, and 5 ug/ml forskolin. Chase media for PCNA and pcNGFR+ cells contained high-glucose DMEM, 10% FCS and HAT. In experiments involving tunicamycin, cells were preincubated in media containing 10 ug/ml tunicamycin for 100 minutes; the same tunicamycin concentration was maintained in the labelling and chase media. Following the chase period, conditioned media from all cultures were collected and stored on ice. Cells were solubilized in 1.5 ml of RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% deoxycholate, 0.5% NP-40, 0.1% SDS) containing 1 ug/ml aprotinin and 10 uM PMSF on ice for 45 minutes, transferred to precooled tubes and vortexed for 20 seconds. Conditioned media were supplemented with a solution of NP-40 and SDS to produce final concentrations equivalent to that in RIPA.

Labelling of Cell Surface Proteins

Cell surface labelling with Na[^{125}I] was carried out essentially as described (Harlow and Lane, 1989). Nearly confluent cultures of Schwann cells in 100 mm plates were rinsed once with PBS and 0.5 ml PBS was added to each dish. Lactoperoxidase (0.1 U), 500 uCi of Na[^{125}I] and 1 ul of 30% H_2O_2 diluted 1:20000 were added to each dish and additional H_2O_2 was added at 1 minute intervals over the next 4 minutes. The reaction was terminated by the addition of 1 mM dithiothrietol and 1 mg/ml tyrosine in 0.5 ml PBS. Cells were rinsed

twice with PBS and dissolved in RIPA for immunoprecipitation.

Immunoprecipitation

Cell lysates and conditioned media were cleared by centrifugation in a microfuge for 15 minutes at 4° C. Supernatants were collected and exposed to anti-mouse IgG_1 agarose beads (Sigma) for 15 minutes at 4° C with rotation to remove proteins that stick nonspecifically. Beads were removed by centrifugation for 1 minute at 4° C in a microfuge. Equal aliquots of each supernatant were incubated with 10 ug of either MC192, p3 or MOPC21 for 60 minutes on ice followed by incubation with anti-mouse IgG_1 or anti-rabbit IgG agarose beads (Sigma) for 30 minutes at 4° C with rotation. Beads were pelleted by a 60 second microfuge spin and washed 4 times with RIPA at 4° C. Washed beads were suspended in 25 ul of SDS-PAGE sample buffer, boiled 5 minutes and centrifuged for 10 minutes prior to electrophoresis. The supernatants were analyzed by electrophoresis on SDS-PAGE.

Electrophoresis

Samples from Schwann cells that were directly crosslinked with [^{125}I]NGF were analyzed on 7-15% gradient SDS-PAGE (Laemmli, 1970); samples from the metabolic labelling studies were analyzed by 10% linear SDS-PAGE using a minigel apparatus (Hoeffer). All gels were fixed in 20% methanol and 10% acetic acid and treated with Enhance (NEN) according to the manufacturers instructions. After drying, gels were exposed to Kodak XAR film at -70° C.

Hybridization Probes

For Southern blot analysis and RNase protection assays, a 545 base pair Bam

HI/Stu I fragment containing nucleotides 700-1245 of the rat NGF receptor cDNA (Radeke et al. 1986)(kindly donated by Dr. Moses Chao, Cornell University Medical College) was subcloned into pGEM3Z (Promega) and radiolabelled antisense cRNA probes were transcribed with T7 polymerase (BRL) and [^{32}P]CTP (800 Ci/mmol; NEN) using conditions described previously (Melton et al. 1984). For RNase protection assays, template DNA was removed at the completion of transcription by digestion with 5 U RNase-free DNase (Boehringer-Mannheim) in the presence of 20 U RNasin (Promega) in a total volume of 25 ul. Reactions mixtures were extracted once with phenol/chloroform, supplemented with sodium acetate and ethanol and precipitated at -80° C.

RNA Preparation and RNase Protection Analysis

Total RNA was isolated from nearly confluent primary rat Schwann cell cultures and PC12 cells using the guanidine hydrochloride method. For RNase protection assays, 5 ug of total RNA was combined with approximately 10^6 cpm of labelled cRNA in 180 ul of hybridization buffer containing 80% formamide, 40 mM Pipes (pH 6.4), 0.4 M NaCl and 1 mM EDTA, heated to 90° C for 2 minutes, and transferred to a 55° C water bath for 16 hours. Samples were supplemented with 300 ul 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA containing 40 ug/ml RNase A (Boehringer-Mannheim) and 1200 U/ml RNase T1 (BRL) and incubated for 30 minutes at 30° C. SDS and proteinase K were added to final concentrations of 0.4% and 100 ug/ml respectively and tubes were incubated at 37° C for 20 minutes. The samples were phenol/chloroform extracted, precipitated with isopropanol in the presence of carrier tRNA, and electrophoresed on a denaturing acrylamide gel (6% acrylamide, 8 M urea, 1X TBE).

PCR and Southern Blot Analysis

Total Schwann cell RNA (5 ug) was reverse transcribed with Superscript MMLV endo H⁻ reverse transcriptase (BRL) in a 50 ul reaction containing 10 mM Tris-HCl (pH 8.0), 1.25 mM deoxynucleotides, 10 mM dithiothreitiol, 50 mM KCl, 15 mM $MgCl_2$, 0.02% gelatin and 50 pmol primer BA9 for 60 minutes at 42° C. The reaction mixture was diluted with H_2O to a final volume of 1 ml and 5 ul was used for each PCR analysis. PCR was performed in 50 ul reaction volumes using 15 pmol each of primer BA4 with BA10, BA7 with BA8, BA7 with BA10 or BA8 with BA13 in the presence of 1.25 mM deoxynucleotides, 1.25 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 0.01 M dithiothreitiol, 50 mM KCl, 0.02% gelatin and 1.25 U Taq polymerase (BRL) in a GENE-ATAQ thermal cycler (Pharmacia/LKB) programmed for 25 cycles of 40 seconds at 95° C, 1 minute at 60° C and 3 minutes at 72° C. Perfect-Match Polymerase Enhancer (1 U; Stratagene) was included in PCR using primer BA7 with BA10. PCR primers are listed:

BA4 5'-CAG CTC CGG CGG GCA GCA GGC GCT GGA GCG-3'

BA7 5'-CAG CGT GTG CGA GGT GGG CTC GGG ACT CGT-3'

BA8 5'-GGA ATT CAG AGG CCC TGC ACA GAG ATG CTC CGT TC-3'

BA9 5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TTT TT-3'

BA10 5'-GAC TCG AGT CGA CAT CG-3'

BA13 5'-GAA TTC CTC CAG AGC AAG ACC TT-3'

PCR products were separated on 1% agarose TBE gels, transferred to Nytran (Schleicher and Schuell) with 10X SSC and hybridized overnight at 55° C with approximately 10^7 cpm labelled cRNA in a solution of 50% formamide, 40 mM PIPES (pH 6.8), 0.6 M NaCl, 20 mM EDTA, 0.5% SDS, 0.5 mg/ml sheared salmon sperm DNA, 0.2 mg/ml yeast tRNA and 5X Denhardt's solution. Membranes were washed once at room temperature in 2X SSC, 0.1% SDS, twice for 30 minutes at

66° C in 2X SSC, 0.1% SDS and twice for 30 minutes at 66° C in 0.1X SSC, 0.1% SDS. Membranes were exposed to Kodak XAR film at -70.

DNA Sequencing

DNA sequencing of double-stranded pGEM4Z templates (Promega) was performed using the dideoxy-chain termination method (Sanger et al. 1977) modified for the use of T7 DNA polymerase as described by the manufacturer (Sequenase).

RESULTS

Intact and truncated NGF receptor production by cultured Schwann cells

The intact and truncated forms of the NGF receptor were detected on Schwann cell extracts and in conditioned media, as previously reported (DiStefano and Johnson, 1988). Figure 1 shows that [^{125}I]NGF crosslinked to the intact surface receptor on Schwann cells and immunoprecipitated with MC192 has apparent molecular masses of 100 and 220 kd on SDS-PAGE (lane C). The 220 kd species is believed to be an aggregate of the 100 kd species (Buxser et al. 1983). Both species were fully competed when crosslinking was done in the presence of a 250 fold excess of unlabelled NGF (lane D). Parallel experiments in which [^{125}I]NGF was crosslinked to proteins in media conditioned by Schwann cells for 72 hours identified the soluble NGF receptor, with an apparent molecular mass of 65 kd (lane A). Assuming that a single monomeric subunit of [^{125}I]NGF (13.2 kd) is incorporated into each of these complexes, the predicted apparent molecular mass of the membrane-bound and soluble forms are approximately 85 and 50 kd, respectively, in agreement with values published previously.

Generation of the intact and truncated forms of the NGF receptor

To determine whether the truncated and intact forms of the NGF receptor are co-synthesized within Schwann cells, we carried out pulse chase experiments, monitoring the incorporation of [^{35}S]cysteine into protein that could be immunoprecipitated with antibodies to the NGF receptor. To identify NGF receptor protein, we treated cell lysates with one of two antibodies to the NGF receptor. p3 is a polyclonal antibody raised against a synthetic

peptide corresponding to a highly conserved intracellular domain of the NGF receptor in chick. The second antibody, MC192, reacts with the extracellular domain of the rat receptor (Chandler et al. 1984). To ensure that both antibodies recognize the same molecule, we treated lysates of labelled Schwann cells that had been chased for 5 hours first with MC192 or with MOPC21, a nonspecific mouse IgG_1, and subsequently with p3. Figure 2 shows that the p3 antibody was unable to immunoprecipitate radiolabelled protein from lysates previously treated with MC192. However, in lysates treated with MOPC21, p3 precipitated a protein that migrated on SDS-PAGE with identical mobility to the molecule immunoprecipitated by MC192. Therefore, MC192 and p3 recognize the same molecule in Schwann cell lysates.

For pulse chase experiments, Schwann cells were incubated in media containing [^{35}S]cysteine for 30 minutes, chased with media containing unlabelled cysteine for up 120 minutes and immunoprecipitated with either MC192 or the p3 antibody. Figure 3A shows that directly after labelling, the p3 antibody recognizes an 80 kd species. By 30 minutes, two species (82 and 85 kd) are detectable with the p3 antibody, and at the 60 minute and 120 minute time points, levels of the 85 kd species appear to increase at the expense of the 82 kd protein, suggesting a precursor-product relationship. The shift from the 82 kd to the 85 kd form was relatively slow, and 82 kd protein was still detectable after a four hour chase[2]. Results with MC192 differed; this antibody failed to recognize the 80 kd species precipitated by the p3 antibody, but did recognize the other two species following 30, 60, and 120 minute of chase. Although p3 appeared to recognize all NGF receptor species

[2]unpublished observation

equally, MC192 appeared to precipitate the 85 kd species more effectively than the 82 kd form. No proteins with molecular weights in the range of the truncated form of the NGF receptor were detected (Figure 3A), suggesting that the soluble form of the receptor is not synthesized de novo.

To determine which of these NGF receptor species reached the cell surface, we performed cell surface labelling with Na[^{125}I]. Using MC192 or p3, we could immunoprecipitate only the 85 kd protein from lysates of surface-labelled Schwann cells (Fig. 3B) suggesting that the 80 and 82 kd forms are incompletely processed forms of the NGF receptor that are not present on the cell surface.

As yet we do not know how the 80 kd, 82 kd, and 85 kd molecules differ chemically or what accounts for differences in their reactivity to MC192. To test if N-linked glycosylation of the receptor could be involved, we carried out pulse-chase experiments in cells treated with tunicamycin, an antibiotic that blocks formation of N-linked carbohydrate. As previously noted for the human NGF receptor (Grob et al 1985), rat NGF receptor synthesized in tunicamycin-treated cells has a lower apparent molecular mass than receptor synthesized in the absence of the drug (Fig. 4). Nonetheless, although the amount of NGF receptor immunoprecipitated from tunicamycin-treated cells was reduced relative to controls, the receptor was recognized by both p3 and MC192, suggesting that N-linked carbohydrate is not required for recognition by MC192.

To monitor the synthesis of the truncated form of the NGF receptor and to compare the kinetics of its production with that of the intact receptor, we repeated the pulse chase experiments over a longer time period, analyzing both cell lysates and conditioned media. For these studies, immunoprecipitations

were done solely with MC192 since the p3 antibody does not recognize the truncated form of the receptor (data not shown). This result is not surprising since the p3 antibody was raised against the intracellular portion of the receptor which is likely absent in the truncated molecule. Figure 5A shows that in cell lysates, newly synthesized receptor protein was precipitated 1 to 30 hours after initiating the chase, with levels peaking by 5 hours, and remaining relatively stable for 20 hours. Some reduction in levels of immunoprecipitated protein were evident by 30 hours. By comparison (Fig. 5B), the truncated NGF receptor (M_r 50 kd) in conditioned medium was not evident until 5 hours after chase and increased in concentration to a maximum by 20 hours. At the 20 and 30 hour time points, an additional, slightly larger immunoprecipitation product (M_r 52 kd) was also evident. MOPC21, a non-specific monoclonal antibody, did not precipitate either molecule.

Only a single mRNA is produced from the NGF receptor gene

Our results show that the truncated form of the NGF receptor is undetectable in radiolabelled cell lysates and that it appears in conditioned medium well after the intact receptor is produced intracellularly, strongly suggesting that the truncated receptor is generated from the intact molecule as a result of post-translational processing. However, an alternative explanation could be that the truncated receptor is synthesized *de novo* from a mRNA splice variant that encodes a protein not recognized in cell lysates by MC192. We have tested this possibility directly. The polymerase chain reaction (PCR) was employed to detect NGF receptor splice variants. The strategy employed, shown in figure 6A, is based on the hypothesis that potential splice variants encoding the truncated NGF receptor would differ from mRNA encoding

the intact receptor across the transmembrane domain. Primers were employed to amplify sequences between nucleotides 758 (BA13) to 1526 (BA8), 491 (BA7) to 1526 or 1 (BA4) to 1526. In each case, fragments of appropriate sizes (768, 1036, and 1527 base pairs, respectively) were obtained (Fig. 6B). Cloning and sequencing of the smaller cDNAs in lanes A and B revealed that they were products of aberrant priming by primer BA8 beginning at nucleotide 1053.

To identify NGF receptor gene products with unique 3' ends, we performed PCR between nt 491 and an oligo d(t) based primer. This analysis revealed a major product of about 3.1 kb as well as several smaller products (Fig. 6B). When analyzed by Southern blotting, digestion of these PCR products with Pvu II showed that virtually all were identical to bona fide NGF receptor cDNA between nucleotides 491 and 1274. Therefore, these subsidiary bands likely resulted from mispriming events. Bands that did not digest appropriately accounted for only a small fraction of the total. One such species was subcloned and sequenced and found to represent an unusual PCR artifact[3] but no evidence of alternate splicing was found.

To confirm the PCR analysis, we carried out RNase protection assays using a 573 base riboprobe containing 28 nucleotides of vector sequence and RNA complementary to nucleotides 701-1246 of the NGF receptor. This portion of the mRNA encodes the last 55 amino acids of the extracytoplasmic domain, the entire transmembrane domain and 104 amino acids of the cytoplasmic domain. When performed on total RNA isolated from either Schwann or PC12 cells, we detected only a single protected band of 545 nucleotides (Fig. 7). No signal was obtained from control reactions containing tRNA.

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[3]P.A. Barker, F.D. Miller, and R.A. Murphy, manuscript submitted

Truncated NGF receptor is produced post-translationally in transfected cell lines.

To show definitively that truncation of the NGF receptor can occur post-translationally, we carried out pulse-chase studies on mouse LTK$^-$ fibroblasts transfected with either the intact NGF receptor gene (PCNA cells) or the NGF receptor cDNA (pcNGFR$^+$). Since NGF receptor mRNA produced within the pcNGFR+ cells cannot undergo splicing, truncated NGF receptor could only be produced by post-translational processing. Following pulse chase, cell lysates and conditioned media were immunoprecipitated with MC192. Figure 8 shows that lysates from both cell types contained abundant NGF receptor. Furthermore, truncated receptor was present in media conditioned by both cell types. Both the surface bound and soluble forms were considerably more abundant in cultures of PCNA than pcNGFR$^+$ cells. The intact and soluble receptors produced by pcNGFR$^+$ cells migrated more slowly than those produced by PCNA cells. The difference is probably due to increased glycosylation of the extracellular domain; comparison of genomic sequence with that of the cDNA used to transfect the pcNGFR$^+$ cells has shown that the latter contains an extra N-linked glycosylation consensus site in its extracellular domain[4].

DISCUSSION

Several lines of evidence presented in this study suggest that the truncated form of the NGF receptor is generated in primary rat Schwann cells as a result of post-translational processing. Pulse chase labelling followed

[4]Dr. S.O. Meakin, Stanford University, personal communication

by immunoprecipitation analyses using antibodies to the NGF receptor reveal that proteins with molecular weights in the range of the intact receptor (80-85 kd) are detectable in Schwann cell lysates within minutes of metabolic labelling and remain detectable for at least 30 hours. During the same time period, no proteins comparable in size to the truncated form of the receptor could be detected intracellularly. The truncated receptor did appear in conditioned media at much later time points, between 5 and 10 hours post-labelling, and was detectable for at least an additional 20 hours. Also, cells transfected with NGF receptor cDNA, which cannot be spliced, produce both the intact and truncated forms of the receptor. Taken together, these results suggest that the truncated form of the NGF receptor is generated from the intact protein by post-translational processing. Proteolysis of intact receptor has been postulated to explain the generation of soluble forms of the interleukin-2 (Loughnan et al. 1988) and polymeric immunoglobulin receptors (Mostov and Simester, 1985) from their respective membrane-anchored precursors. It should be noted that we detected two forms of truncated NGF receptor that differ in molecular mass (50 and 52 kd) and in their rate of production. This result suggests that NGF receptors on Schwann cells may be the target of more than one proteolytic activity.

Alternative splicing produces mRNAs that encode a number of truncated, soluble forms of receptors, including those for interleukin-4 (Mosley et al. 1989), interleukin-6 (Novick et al. 1989), human chorionic gonadotrophin (Loosfelt et al. 1989), and GM-CSF (Ashworth and Kraft, 1989). Our results indicate that an NGF receptor splice variant is not involved in generation of the truncated NGF receptor. First, the slow rate of appearance of the truncated receptor following metabolic labeling is not consistent with its

being translated de novo. Furthermore, our PCR and RNase protection analyses of the portion of the NGF receptor mRNA encoding the extracellular and transmembrane domains revealed no splice variants or novel transcripts that could encode a truncated protein. These results are in agreement with previous S1 nuclease assays in which probes specific for extracellular and intracellular and 3' untranslated (Hempstead et al. 1988; Buck et al. 1988) portions of the NGF receptor cDNA failed to detect any evidence of alternative splicing.

While only one mRNA appears to code for the NGF receptor, our data suggest that formation of the intact receptor protein occurs in a multistep process that can be dissected by domain-specific antibodies. The p3 antibody, which is specific for an intracellular portion of the protein, detected an 80 kd form of the receptor immediately after labelling that was not recognized by MC192. During the chase period, this species was replaced by 82 kd and 85 kd molecules, which appeared to have a precursor/product relationship, and which were recognized by both p3 and MC192 antibodies. MC192, which reacts with the extracellular portion of the molecule, recognized the 85 kd form better than the 82 kd form for reasons unknown. The 82 kd form of the protein converts to the 85 kd form relatively slowly and is still detectable intracellularly four hours after chase (data not shown). This suggests that early post-translational events such as signal peptide removal and disulfide bond formation are unlikely to be involved in this transition and instead favors changes that occur within the Golgi apparatus. Addition of N- and O-linked sugars may be involved since both are present within the extracellular domain of the mature protein (Zupan et al. 1989). Welcher et al. (1991) have shown that cloned forms of the receptor which lack the serine and threonine rich

membrane-proximal region of the extracellular domain still bind MC192 but that receptors lacking the N-terminal domain, which include the sites of N-linked glycosylation, do not. In our studies, NGF receptor from tunicamycin-treated cells migrated with a reduced molecular weight, as reported previously for the human NGF receptor (Grob et al. 1985), but was still immunoprecipitated by MC192, suggesting that N-linked sugars may not be essential for antibody recognition. The dependence of MC192 binding on post-translational processing raises the possibility that within some cells, NGF receptors may be processed in such a way that they are not reactive with this antibody. Other monoclonals directed against the NGF receptor may react differently. ME20.4, a monoclonal antibody directed against the extracellular domain of the human NGF receptor, has recently been shown to bind unglycosylated receptor produced in a baculovirus expression system (Vissavajjhala and Ross, 1990).

Despite the fact that truncated, soluble forms of membrane receptors for a number of protein ligands have been identified, their physiological roles are unknown. The truncated IL-4 receptor neutralizes the growth promoting activity of IL-4 <u>in vitro</u> (Mosley et al. 1989) and systemic administration of the truncated IL-1 receptor nullifies the action of Il-1 <u>in vivo</u> (Fanslow et al. 1990). Since the ligand binding domain of the truncated NGF receptor binds NGF as well as the low affinity membrane bound form of the receptor (Vissavajjhala and Ross, 1990; Welcher et al. 1991), it too has the potential to compete for ligand and to inhibit biological activity. Alternatively, the soluble truncated NGF receptor may serve as a carrier protein that plays a role in the extracellular transport of NGF between cells or in affecting its rate of proteolysis or clearance. It should be noted that the truncated NGF receptor may exert physiological effects not only upon the action of NGF but

also on related neurotrophins. Brain-derived neurotrophic factor, which shares about 55% amino acid identity with NGF (Leibrock et al. 1989), binds to the low affinity NGF surface receptor with affinity equal to that of NGF (Rodriguez-Tabar et al. 1990) and is likely to bind to the truncated NGF receptor as well. Also, NGF binding proteins that may be unrelated to the truncated NGF receptor have been identified in medium conditioned by NGF-producing mouse fibroblasts (Siminoski and Murphy, 1987). Determining the role of neurotrophin binding proteins may reveal novel aspects of neurotrophin function.

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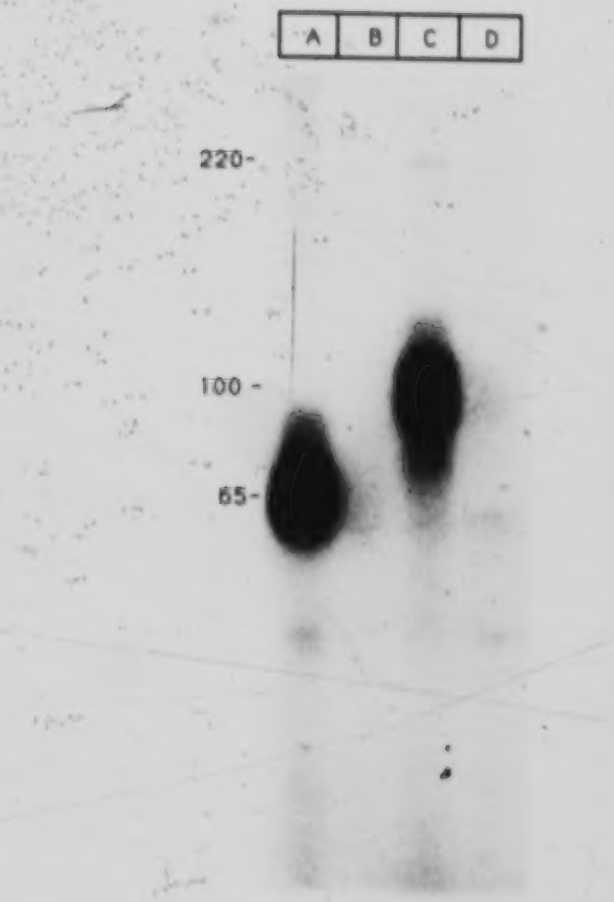
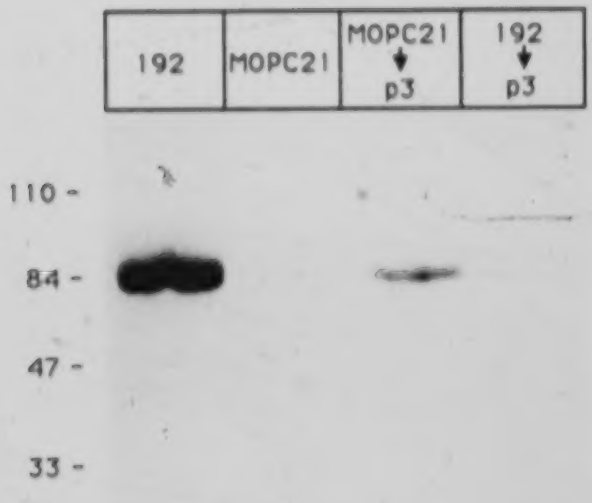
FIGURES - CHAPTER 1

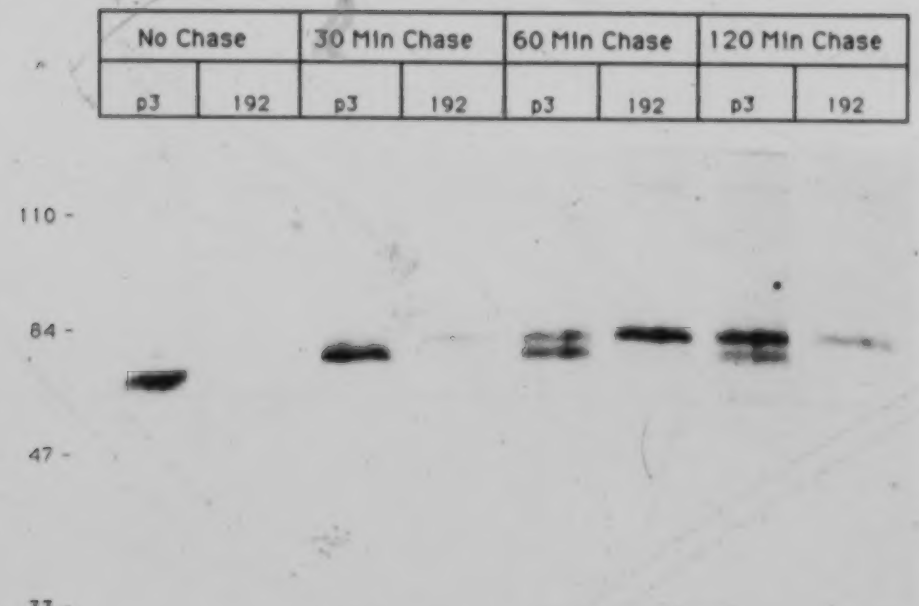
Fig. 1 Detection of intact and truncated NGF receptor derived from rat Schwann cells. Schwann cells (lanes C and D) or Schwann cell conditioned media (lanes A and B) were incubated with ^{125}I-NGF, crosslinked with EDAC, immunoprecipitated using MC192 and analyzed by SDS-PAGE followed by fluorography. In lanes B and D, samples were incubated with ^{125}I-NGF in the presence of a 250-fold excess of unlabelled NGF.

Fig. 2 Confirmation that MC192 and p3 antibodies detect the same molecule. Schwann cells metabolically labelled 30 minutes with ^{35}S-cysteine and chased five hours were solubilized in RIPA and extracts were immunoprecipitated with either MC192 or MOPC21, followed by an additional immunoprecipitation with p3, a rabbit polyclonal antibody directed against an NGF receptor peptide. Labelled proteins were analyzed by SDS-PAGE and fluorography.


A
B
C
D
220-
100 -
65-


192
MOPC21
MOPC21
p3
192
p3
110 -
84 -
47 -
33 -

Ffg. 3 Analysis of intracellular and surface bound Schwann cell derived NGF receptor. (Top panel) Schwann cells were metabolically labelled with [^{35}S]cysteine, chased with unlabelled cysteine for the times indicated and NGF receptor immunoprecipitated from cell lysates using either p3 or MC192. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. (Bottom panel) Intact Schwann cells were surface labelled with [^{125}]I using lactoperoxidase, solubilized and immunoprecipitated with either MC192, MOPC21, p3 or non-specific rabbit IgG (NSR). Labelled proteins were analyzed by SDS-PAGE and fluorography.


No Chase
30 Min Chase
60 Min Chase
120 Min Chase
p3
192
p3
192
p3
192
p3
192
110 -
84 -
47 -
33 -

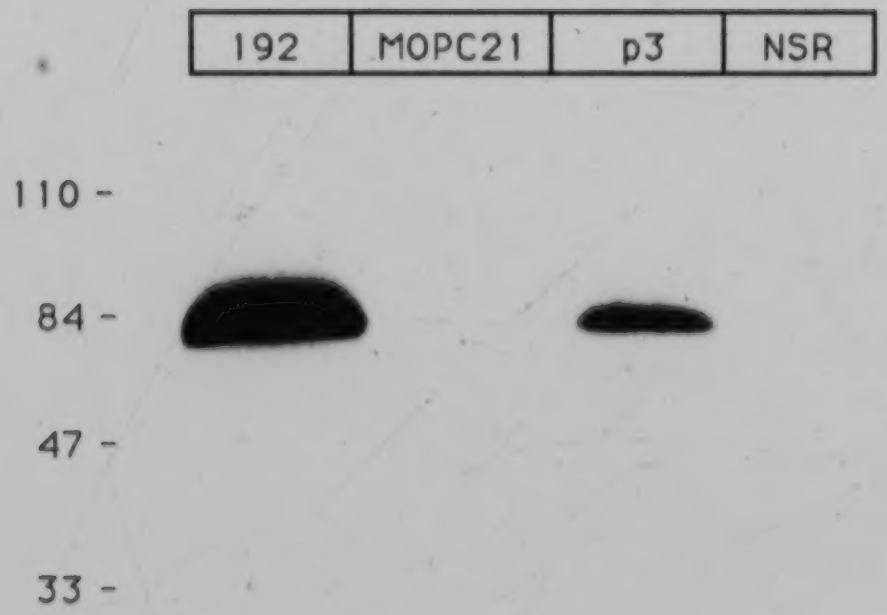
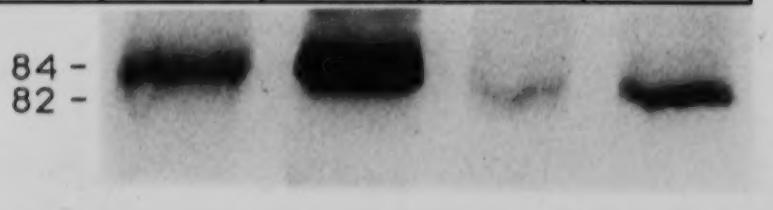

192
MOPC21
p3
NSR
110 -
84 -
47 -
33 -

Fig. 4 **Effect of tunicamycin treatment on NGF receptor immunoreactivity.** Schwann cells were pretreated with tunicamycin (10 ug/ml) and then metabolically labelled and chased for an additional 5 hours in the presence of the drug. Cell lysates were immunoprecipitated with either MC192 or p3. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Fig. 5 **Pulse chase analysis of NGF receptor in cell lysates and in conditioned media.** Schwann cells were metabolically labelled with ^{35}S-cysteine and chased with cold cysteine for the time periods indicated. Cell lysates (panel A) or conditioned media (panel B) were immunoprecipitated with MC192 and analyzed by SDS-PAGE and fluorography. Immunoprecipitations with non-specific MOPC21 were carried out on samples chased for 30 hours.

Antibody	p3	192	p3	192
Tunicamycin	-	-	+	+



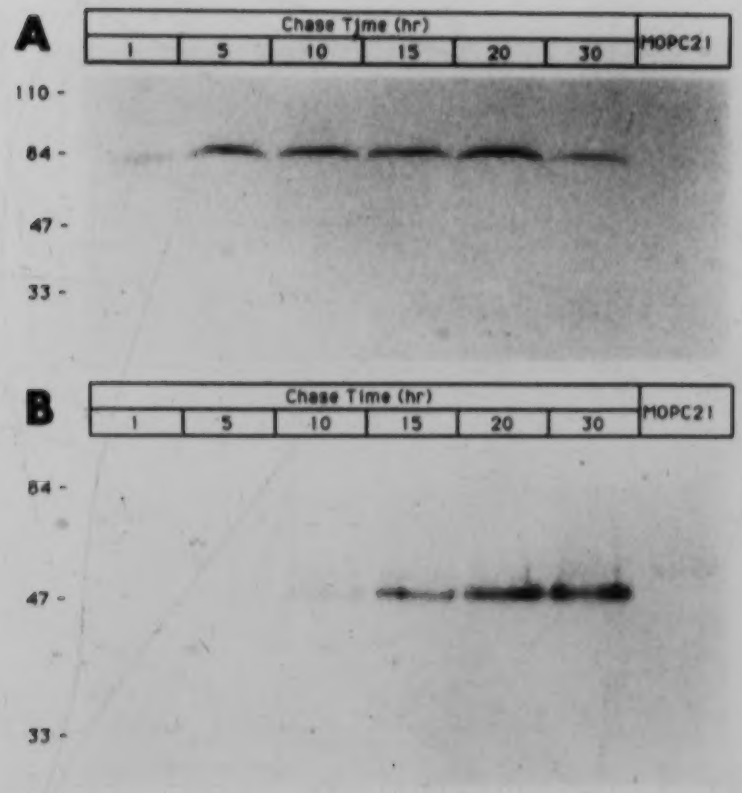
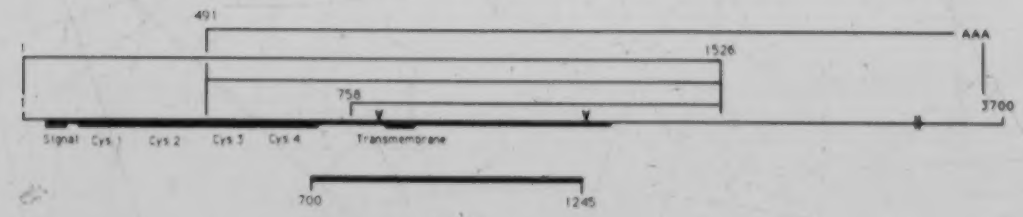


Fig. 6 **PCR analysis of NGF receptor mRNA.** (Top) Strategy employed for PCR analysis. The open reading frame of NGF receptor is indicated by heavy solid lines, 5' and 3' untranslated regions are indicated by light solid lines, arrowheads indicate the position of PvuII sites and the heavy solid line at the bottom of the figure (700 - 1245) indicates that the cRNA probe employed for Southern blot analysis and RNase protection assays. The upper thin solid lines indicate the various cDNA regions amplified by PCR. (B) NGFR cDNA between nt 758 and 1526 (primers BA13 and BA8 (lane A)), nt 491 to 1526 (primers BA7 to BA8 (lane B)), nt 1 and 1526 (primers BA4 to BA8 (lane C)) and nt 491 to the poly(a) tail (primers BA7 to BA10 (lane D)) were amplified from Schwann cell cDNA using PCR as described in Materials and Methods. In lane E, cDNA produced by PCR amplification using primers BA7 and BA10 was digested to completion with PvuII. All PCR products were subjected to electrophoresis on 1% TBE gels, transferred to nitrocellulose, and analyzed by Southern blotting using [^{32}]P-labelled cRNA encoding nt 700 to 1250 of the NGF receptor sequence.


491
AAA
1526
758
3700
Signal
Cys 1
Cys 2
Cys 3
Cys 4
Transmembrane
700
1245

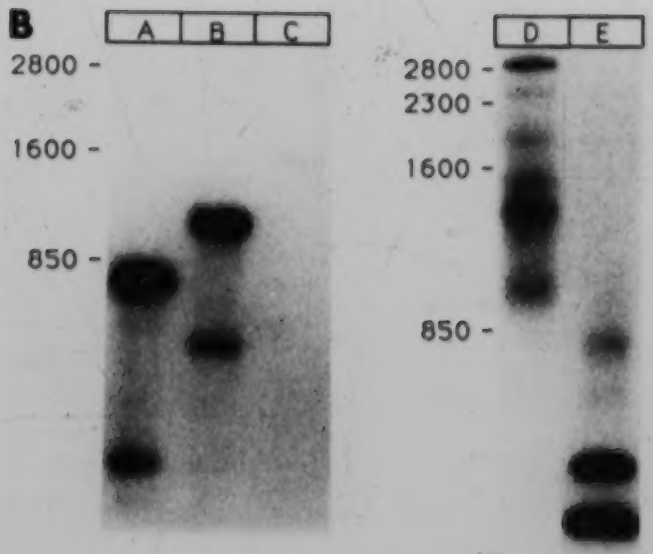
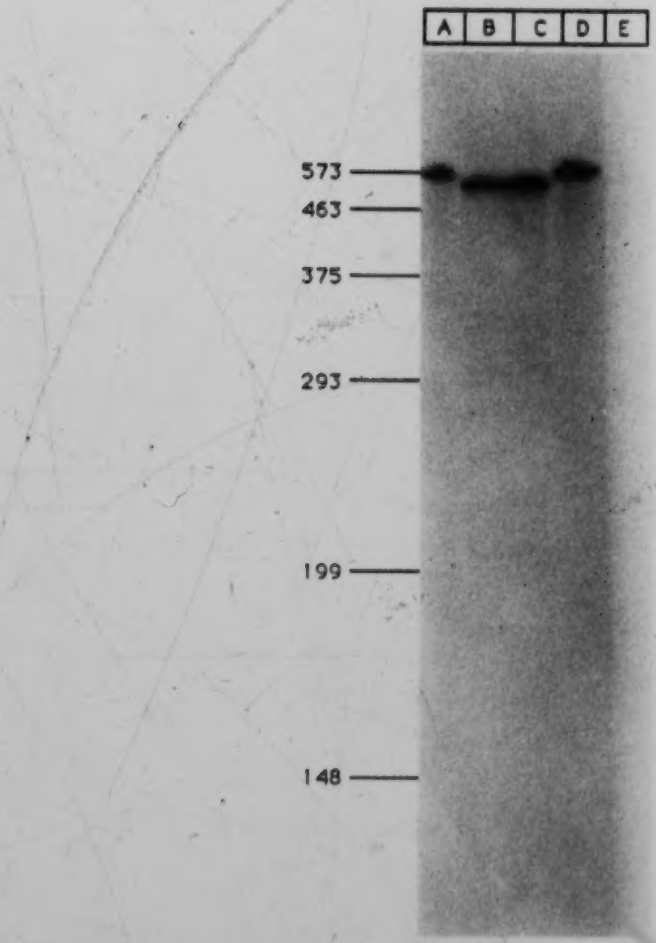

B
A
B
C
2800 -
1600 -
850 -
D
E
2800 -
2300 -
1600 -
850 -

Fig. 7 **RNase protection analysis of NGF receptor mRNA.** A [^{32}]P-labelled cRNA complementary to nt 700 to 1250 of the rat NGF receptor mRNA was used in RNase protection assays as described in the Materials and Methods. Assays were performed on 5 ug total RNA isolated from PC12 cells (lane B) and Schwann cells (lane C) and on 20 ug yeast tRNA (lane E). Lanes A and D contain intact undigested cRNA.

Fig. 8 **Immunoprecipitation analysis of NGF receptor-transfected cell lines.** PCNA cells (lanes A, C and E) or pcNGFR+ cells (lanes B, D and F) were metabolically labelled for 5 hours. Cell lysates (lanes A to D) or conditioned media (lanes E and F) were immunoprecipitated with either MC192 (lanes A, B, E and F) or MOPC21 (lanes C and D). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.


A
B
C
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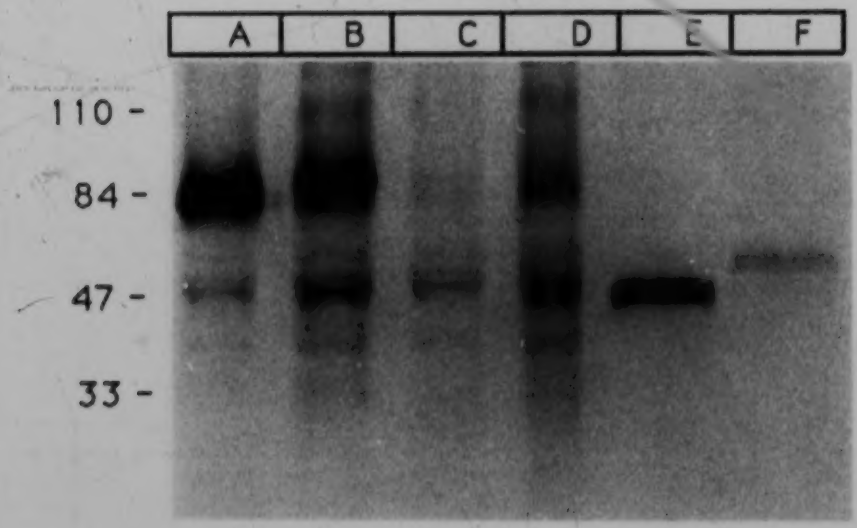
E
573
463
375
293
199
148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ANALYSIS OF NGF RECEPTOR GENE PRODUCTS: GENERATION OF ARTEFACTUAL SPLICE VARIANTS BY PCR.[1]

The polymerase chain reaction (PCR) has proven to be a particularly useful technique for characterizing mRNA transcripts since it can detect both rare alternative splicing events and provide sufficient material for cloning or direct analysis. However, in several instances, the simple presence of PCR products which do not correspond to the known mRNA species has been given as the only evidence for the existence of alternatively spliced mRNA species (Doorbar et al. 1990; Rotenberg et al. 1989; Llorens-Cortes et al. 1990). We would like to draw attention to the fact that PCR amplification products derived from reverse transcribed RNA may contain artefacts that are not readily apparent. We have recently used PCR together with RNase protection assays to characterize products of the nerve growth factor (NGF) receptor gene and found that, unless rigorously controlled, these two techniques can generate misleading results.

These results arose in the course of studies that were designed to determine whether the truncated form of the NGF receptor, which is a soluble protein that maintains ligand binding capability (DiStefano and Johnson, 1988), arises from proteolytic cleavage of intact receptors or from de novo synthesis of alternatively spliced mRNA. To test the latter alternative, we used PCR to perform an extensive analysis of NGF receptor mRNA isolated from primary Schwann cells. Using the RACE protocol (Frohman et al. 1988) to identify transcripts with unique 3' ends, we identified the authentic NGF receptor transcript (Radeke et al. 1986) as well as a possible splice variant.

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1. A version of this chapter has been submitted to Biotechniques.

Cloning and sequencing of this latter cDNA revealed that it contained NGF receptor sequences 5' to nt 905 contiguous with NGF receptor sequences 3' to nt 2763 (data not shown). Generation of this product was reproducible under a variety of different PCR conditions. The genomic structure of the rat NGF receptor gene has not yet been determined but examination of the NGF receptor cDNA revealed that the 5' junction of this putative splice variant conformed to the splicing consensus, but that the 3' junction did not, suggesting that, if authentic, the 3' junction must be adjacent to an intron.

To test the authenticity of the putative splice variant, we used the novel PCR clone for RNase protection assays on RNA isolated from cells known to contain NGF receptor mRNA (Figure 1, lanes A-C). As expected, fragments of the cRNA corresponding to the noncontiguous 5' (199 nt) and 3'(292 nt) portions of the previously characterized NGF receptor mRNA were protected. In addition, the entire 491 nt cRNA was also protected, indicating that RNA complementary to the putative splice variant was actually present within these samples.

Although these experiments strongly suggested the presence of an NGF receptor splice variant, subsequent pulse chase analysis of the truncated NGF receptor indicated that it is actually formed post-translationally (Barker et al. submitted). We therefore performed several additional control experiments which led us to conclude that the splice variant was an artefact. First, we performed PCR on Schwann cell cDNA using primers that flanked the putative splice junction, and detected only bona fide NGF receptor mRNA. Second, we performed RNase protection with a probe containing nucleotides 700 to 1250 (Figure 1, lanes D-H), and did not detect a fragment (206 nt) corresponding to the splice variant. Third, we used PCR to isolate the genomic sequence

flanking the 3' putative splice donor and found that no intron was present. Taken together, these data indicate a) that the putative splice variant was the result of an artefact introduced either during reverse transcription or PCR and b) that the RNase protection assay using this artefactual product as template produced misleading results. To prove this, we synthesized sense strand NGF receptor RNA in vitro and tested it in protection assays. Protected fragments of 199, 292 and 491 nt were observed, firmly establishing that the 491 bp labelled fragment was protected by noncontiguous sequences.

PCR is currently widely used for detection of splice variants. In several cases, the sole evidence for their existence arises from the demonstration of amplification products that differ in size from the expected cDNA. Our results indicate that PCR can reproducibly produce artefactual "splice variants" and show that similar results obtained using only this technique should be interpreted with caution. Perhaps more surprisingly, our studies also show that direct analysis using an RNase protection assay for the RNA species represented by these PCR products cannot, by itself, be considered a rigorous test. Thus, our data indicate that several different experimental approaches are required before presuming the in vivo existence of apparent splice variants detected using PCR.

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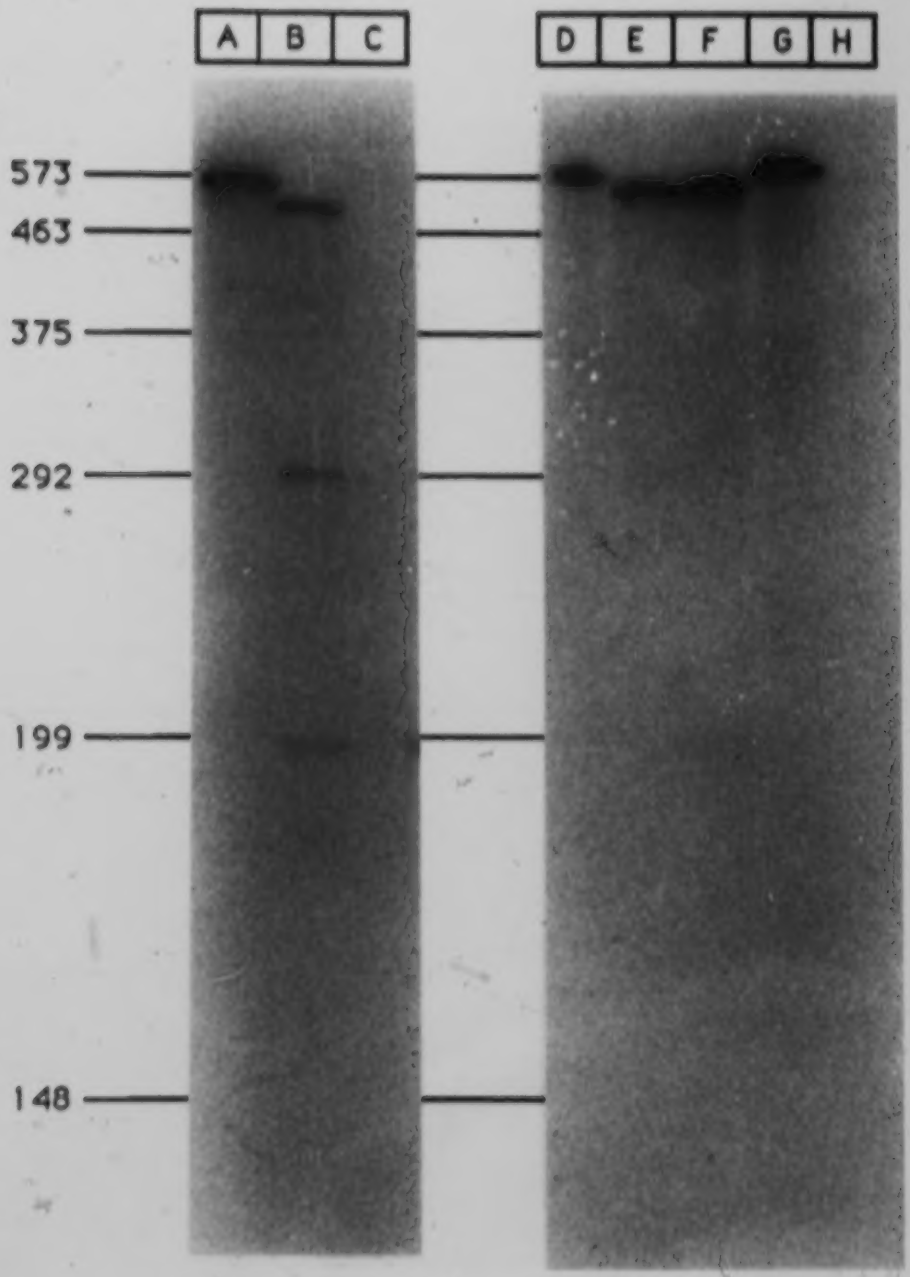
FIGURES - CHAPTER 2

Fig 1 Lanes A-C. RNase protection assays were performed as described (Barker et al. submitted) using cRNA probes generated from the putative splice variant cDNA. Undigested probe is shown in lane A; reactions were performed with 5 ug of total primary rat Schwann cell RNA (lane B) or 20 ug of tRNA (lane C). Lanes D-H. RNase protection assays were performed using cRNA complementary to nt 700 to 1250 of the rat NGF receptor. Undigested probe is shown in lanes D and G; reactions were performed with either 5 ug total PC12 cell RNA (lane E), 5 ug total primary rat Schwann cell RNA (lane F) or 20 ug tRNA (lane H).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Characterization of NGF Receptor Gene Products in Cultured Dermal Fibroblasts

Nerve growth factor (NGF) is required for the growth, differentiation, and maintenance of sensory and sympathetic neurons within the peripheral nervous system (Thoenen and Barde, 1980) and for the survival of cholinergic neurons within the central nervous system (Gage et al. 1990). The effects of NGF are mediated by specific cell surface receptors with two NGF binding components (Type I and Type II) which differ in K_d and dissociation rates (Sutter et al. 1979; Landreth and Shooter, 1980; Schechter and Bothwell, 1981; Bernd and Greene, 1986; Woodruff and Neet, 1986). Only the Type II binding component is present on cells which are unresponsive to NGF (Buxser et al. 1983; Yasuda et al. 1987; DiStefano and Johnson; 1988a; Kasaian and Neet, 1988). The molecular basis of the high affinity Type I NGF receptor remains unclear but the low affinity, fast-dissociating Type II NGF receptor has been well characterized. This 75-85 kd transmembrane glycoprotein has been cloned from rat (Radeke et al. 1986), human (Johnson et al. 1986), and chicken (Large et al. 1989; Heuer et al. 1990). Expression of this molecule within some (Hempstead et al. 1989; Pleasure et al, 1990), but not all (Radeke et al. 1986; Johnson et al. 1986; Reddy et al. 1991) NGF receptor-negative cells produces both Type I and II NGF binding sites as well as functional responses to NGF, suggesting that the low affinity receptor interacts with other cellular components to produce functional NGF receptors. Although these other components remain unidentified, a 158 kd complex identified by NGF crosslinking (Hosang and Shooter, 1985; Meakin and Shooter, 1991) and the trkA proto-oncogene product may be involved (Klein et al. 1991; Kaplan et al. 1991a; Kaplan et al. 1991b). Brain-derived neurotrophic factor, which is

structurally related to NGF but has distinct biological activity (Barde et al. 1982; Leibrock et al. 1989), binds to the Type II NGF receptor with affinity equal to that of NGF (Rodriguez-Tabar et al. 1990), raising the possibility that the Type II receptor may be a component of more than one neurotrophin receptor.

NGF receptor expression during development and in the adult has been examined by direct ^{125}I-NGF binding (Raivich et al. 1985; Verge et al. 1989), by the use of NGF receptor specific monoclonal antibodies (Thompson et al. 1990; Yan and Johnson, 1988; Chesa et al. 1988) and by the use of *in situ* hybridization (Ernfors et al. 1988; Heuer et al. 1990; Byers et al. 1990). These techniques have revealed that a wide variety of non-neuronal tissues and cell types appear to express the NGF receptor gene. Diverse tissues such as developing teeth (Byers, 1990; Byers et al. 1990), somites (Raivich et al. 1985, Ernfors et al. 1988; Heuer et al. 1990) , muscle (Raivich et al. 1985, Ernfors et al. 1988; Heuer et al. 1990) and thymus (Ernfors et al. 1988) appear to express abundant NGF receptor in the developing rat and in adult humans, NGF receptor immunoreactivity has been observed in myoepithelial cells of mammary and salivary glands , in basal epithelium of skin and the oral cavity, within the adventitia of blood vessels and upon the external epidermal sheath of hair follicles (Chesa et al. 1988; Thompson et al. 1989). During development, cells, presumably fibroblasts, within connective tissue express high levels of NGF receptor mRNA (Heuer et al. 1990) and protein (Chesa et al 1988; Byers et al. 1990) and within mouse whisker pads, the developmental pattern of NGF receptor mRNA expression within mesenchymal cells (Wyatt et al. 1990) parallels NGF mRNA expression within the overlying epithelium (Davies et al. 1987).

Why non-neuronal cells express the NGF receptor has not been determined. Johnson and co-workers have suggested that Schwann cells, in response to nerve injury, bind NGF to their surfaces to make the protein available to regenerating neurons which require the protein (Taniuchi et al. 1986), and Byers et al. (1990) have suggested that NGF and its receptor may be involved in paracrine interactions between epithelial and mesenchymal cells during development. However, there have been no biological effects of NGF described for epithelial or mesenchymal cells. In culture, Schwann cells produce mRNA coding for the NGF receptor which is identical to that in PC12 cells (Barker et al. submitted), express Type II NGF receptors on their cell surfaces (Yasuda et al. 1987; DiStefano and Johnson, 1988a) and produce a truncated form of the NGF receptor (DiStefano and Johnson, 1988b), which arises by postranslational processing (Barker et al, submitted). NGF receptors on mesenchymal cells have not been characterized.

In this study we set out to characterize NGF receptors on fibroblasts. Our goal was to determine whether fibroblasts produce authentic NGF receptor mRNA and protein, and, if they do, whether they also respond to NGF. Our results show that dermal fibroblasts in culture synthesize only one form of NGF receptor mRNA which is indistinguishable from that in Schwann cells and PC12 cells. However, unlike PC12 cells and Schwann cells, cultured fibroblasts do not produce detectable levels of NGF receptor protein either in its intact or truncated form. Also, fibroblasts do not respond to NGF as measured by fos expression.

MATERIALS AND METHODS

Tissue Culture

Schwann cells cultures were established essentially as described previously (Barker et al. submitted) and maintained on poly-L-lysine coated tissue culture flasks in DMEM containing 10% fetal calf serum, 10 ug/ml crude glial growth factor (Gospodarowicz, 1975) and 5 ug/ml forskolin. Primary fibroblasts were established as previously described (Acheson et al 1991. submitted) and maintained in DMEM containing 10% fetal calf serum. Experiments involving primary cells were performed on cells between passages 5 and 10. PC12 cells (Greene and Tischler, 1976) were maintained in RPMI media in the presence of 5% fetal calf serum and 10% horse serum.

Antibodies

MC192, an IgG_1 monoclonal antibody specific for the rat NGF receptor (Chandler et al. 1984), was isolated from ascites fluid of mice injected with the appropriate hybridoma (kindly donated by Dr. Eugene Johnson, Washington University) using a Pierce Immunopure kit. MOPC21, a nonspecific IgG_1 mouse monoclonal antibody, was purchased from Organon Technika. The p3 antibody, which is directed against peptide sequence from the intracellular domain of the chicken NGF receptor and which recognizes the rat NGF receptor, has been previously described (Barker et al. submitted).

Animal Surgery and Tissue Fixation

Female Sprague-Dawley rats (175-200 g) were heavily anaesthetized with sodium pentobarbital (65 mg/kg) and shaved with an electric razor on their ventral

surface. For wounding experiments, lesions about 2.5 cm long were made on the shaved ventral aspect using sharp scissors. After 10 hours, during which time the animals remained anaesthetized, skin lying within 3-4 mm of either side of the wound was collected for RNA analysis. Control skin was collected from the ventral aspect of shaved, anaesthetized unlesioned animals. Skin was also collected from the ventral aspect of neonatal rats which had been stunned by a blow to the head and decapitated.

Immunocytochemistry

For immunocytochemistry of cultured fibroblasts, cells grown on 35 mm dishes were rinsed twice with ice-cold PBS then fixed for two minutes in 50% methanol/50% acetone. Nonspecific sites were blocked by incubation for twenty minutes in blocking solution (DMEM containing 5% horse serum and 5% fetal calf serum) and cells were then incubated for one hour at room temperature in blocking solution containing MC192 or MOPC21 (final concentration 10 ug/ml). Cells were washed three times with blocking solution and incubated with a 1/500 dilution of biotinylated anti-mouse IgG (Vector) in blocking solution for 1 hour. Cells were washed three times with serum-free DMEM, incubated with a 1/250 dilution of Texas Red/streptavidin for one hour at room temperature, washed twice with serum-free DMEM and coverslipped.

Equilibrium Binding

The 2.5S form of NGF was isolated as described (Mobley et al. 1976) with some modification (Watson et al. 1985) and radioiodinated by the lactoperoxidase method (Marchalonis, 1969) to specific activities of approximately 60 cpm/pg. Schwann cells or dermal fibroblasts on 35 mm dishes were rinsed twice with

serum-free DMEM and incubated with DMEM containing 0.1% bovine serum albumin (BSA) and 0.1 uM potassium iodide supplemented with various concentrations of ^{125}I-NGF at 4° C for four hours. To remove unbound ^{125}I-NGF, cells were rinsed once with ice-cold phosphate-buffered saline (PBS) containing 0.1% BSA, twice with ice-cold PBS, and solubilized in 0.2 N NaOH, and transferred to fresh tubes for gamma counting. Nonspecific binding was performed as above in parallel dishes containing 0.4 uM 2.5S NGF.

[^{125}I]NGF Crosslinking

Schwann cells or fibroblasts prewashed with phosphate-buffered saline were incubated with 2 nM [^{125}I]NGF in serum-free DMEM for 2 hours on ice. NGF was crosslinked to its receptor by incubation with 20 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) for 15 minutes at room temperature The reaction was terminated by the addition of Tris-HCl (pH 7.5) to a final concentration of 50 mM and cells were solubilized in HEAP buffer (10 mM HEPES, 1 mM EDTA, 1 ug/ml aprotinin, 10 uM PMSF) containing 150 mM NaCl and 1% octylglucoside. ^{125}I-NGF was crosslinked to the truncated form of the NGF receptor in undiluted media which had been conditioned for 72 hours. Parallel reactions containing a 250 fold excess of unlabelled 2.5S NGF were carried out in all experiments to confirm specificity.

Labelling of Cell Surface Proteins

Cell surface labelling with Na[^{125}I] was carried out essentially as described (Harlow and Lane, 1988). Cultures of Schwann cells or dermal fibroblasts in 100 mm plates were rinsed once with PBS and 0.5 ml PBS was added to each dish. Lactoperoxidase (0.1 U), 500 uCi of Na[^{125}I] and 1 ul of 30% H_2O_2 diluted

1:20000 were added to each dish and additional H_2O_2 was added at 1 minute intervals over the next 4 minutes. The reaction was terminated by the addition of 1 mM dithiothrietol and 1 mg/ml tyrosine in 0.5 ml PBS. Cells were rinsed twice with PBS and dissolved in RIPA for immunoprecipitation.

Immunoprecipitation

Cell lysates and conditioned media were cleared by centrifugation in a microfuge for 15 minutes at 4° C. Supernatants were collected and exposed to anti-mouse IgG_1 agarose beads (Sigma) for 15 minutes at 4° C with rotation to remove proteins that adhere nonspecifically. Beads were removed by centrifugation for 1 minute at 4° C in a microfuge. Equal aliquots of each supernatant were incubated with 10 ug of either MC192, p3, MOPC21 or non-specific rabbit IgG for 60 minutes on ice followed by incubation with anti-mouse IgG_1 or anti-rabbit IgG agarose beads (Sigma) for 30 minutes at 4° C with rotation. Beads were pelleted by a 60 second microfuge spin and washed 4 times with RIPA at 4° C. Washed beads were suspended in 25 ul of SDS-PAGE sample buffer, boiled 5 minutes and centrifuged for 10 minutes prior to electrophoresis. The supernatants were analyzed by electrophoresis on SDS-PAGE.

Electrophoresis

Samples from Schwann cells that were directly crosslinked with [^{125}I]NGF were analyzed on 7-15% gradient SDS polyacrylamide gels (Laemmli, 1971); samples from the surface labelling studies were analyzed on 10% linear gels using a minigel apparatus (Hoeffer). All gels were fixed in 20% methanol and 10% acetic acid and treated with Enhance (NEN) according to the manufacturers

instructions. After drying, gels were exposed to Kodak XAR film at -70° C.

Hybridization Probes

For Southern blot analysis, RNase protection assays and Northern blot analysis of NGF receptor gene products, a 545 base pair Bam HI/Stu I fragment containing nucleotides 700-1245 of the rat NGF receptor cDNA (Radeke et al. 1986) was subcloned into pGEM3Z (Promega) and radiolabelled antisense cRNA probes were transcribed with T7 polymerase (BRL) and [^{32}P]CTP (800 Ci/mmol; NEN) using conditions described previously (Melton et al. 1984). For Northern blots of fos mRNA, labelled cDNA was produced by random priming of cDNA encoding the entire rat c-fos open reading frame (Curran et al. 1987).

RNA Preparation, RNase Protection Analysis and Northern Blotting

Total RNA was isolated from primary rat dermal fibroblasts, Schwann cells and PC12 cells by the guanidine hydrochloride method. For RNase protection assays, 5 ug of total RNA was analyzed by RNase protection as previously described (Barker et al. submitted). For Northern blots, 10 ug of RNA was separated on 1% agarose gels containing 1M formaldehyde, transferred to nitrocellulose and baked *in vacuo* for 90 minutes. Blots were prehybridized and hybridized in a solution of 50% formamide, 40 mM PIPES (pH 6.8), 0.6 M NaCl, 20 mM EDTA, 0.5% SDS, 0.5 mg/ml sheared salmon sperm DNA, 0.2 mg/ml yeast tRNA and 5X Denhardt's solution. For Northern blots using cRNA probes, blots were hybridized at 68° C and washed to a stringency of 0.05X SSC at 68° C. For Northern blots using cDNA probes, blots were hybridized overnight at 42° C and washed to a stringency of 0.2X SSC at 68° C.

PCR and Southern Blot Analysis

Total dermal fibroblast and PC12 cell RNA (5 ug) was reverse transcribed with Superscript MMLV endo H^- reverse transcriptase (BRL) in a 50 ul reaction containing 10 mM Tris-HCl (pH 8.0), 1.25 mM deoxynucleotides, 10 mM dithiothreitol, 50 mM KCl, 15 mM $MgCl_2$, 0.02% gelatin and 50 pmol primer BA8 for 60 minutes at 42° C. The reaction mixture was diluted with H_2O to a final volume of 1 ml and 5 ul was used for each PCR analysis. PCR was performed in 50 ul reaction volumes using 15 pmol each of primers BA7 and BA8 in the presence of 1.25 mM deoxynucleotides, 1.25 mM $MgCl_2$. 10 mM Tris-HCl (pH 8.0), 0.01 M dithiothreitiol, 50 mM KCl, 0.02% gelatin and 1.25 U Taq polymerase (BRL) in a GENE-ATAQ thermal cycler (Pharmacia/LKB) programmed for 25 cycles of 40 seconds at 95° C, 1 minute at 60° C and 3 minutes at 72° C. PCR primers are listed:

BA7 5'-CAG CGT GTG CGA GGT GGG CTC GGG ACT CGT-3'

BA8 5'-GGA ATT CAG AGG CCC TGC ACA GAG ATG CTC CGT TC-3'

PCR products were separated on 1% agarose TBE gels, transferred to Nytran (Schleicher and Schuell) with 10X SSC and hybridized overnight at 55° C with approximately 10^7 cpm labelled cRNA in a solution of 50% formamide, 40 mM PIPES (pH 6.8), 0.6 M NaCl, 20 mM EDTA, 0.5% SDS, 0.5 mg/ml sheared salmon sperm DNA, 0.2 mg/ml yeast tRNA and 5X Denhardt's solution. For moderate stringency, membranes were washed once at room temperature in 2X SSC, 0.1% SDS, twice for 30 minutes at 55° C in 2X SSC, 0.1% SDS and twice for 30 minutes at 55° C in 0.1X SSC, 0.1% SDS. For high stringency, the last wash was performed twice in 0.05X SSC at 66° C. Membranes were exposed to Kodak XAR film at -70.

RESULTS

NGF Receptor mRNA is Produced by Dermal Fibroblasts

To determine if the NGF receptor gene was transcribed within cultured fibroblasts, RNA isolated from confluent primary rat dermal fibroblasts and primary rat Schwann cells was assayed for receptor mRNA by Northern blot analysis. Figure 1 shows that cultured Schwann cells contain abundant NGF receptor mRNA with a molecular size of 3.7 kb, as previously reported (Lemke and Chao, 1988). Dermal fibroblasts grown under standard culture conditions also contain significant levels of the 3.7 kb NGF receptor mRNA, although levels were lower than in Schwann cells.

Dermal Fibroblasts Do Not Produce Detectable Levels of NGF Receptor Protein in culture.

To determine if the NGF receptor gene product was expressed on the surface of cultured dermal fibroblasts, MC192, a mouse IgG_1 monoclonal antibody that recognizes the rat NGF receptor, was used for immunocytochemical analysis. Under conditions in which NGF receptors on Schwann cells could be easily visualized either on intact cells or on cells that were permeabilized, in most experiments no specific staining could be identified on fibroblasts, either before or after permeabilization. In a few experiments, some permeabilized fibroblasts contained what appeared to be specific staining within their cytoplasm (Figure 2, panels A and B). However, similar results were not obtained in all cells that were cultured under identical conditions. Therefore, it is difficult to interpret these results.

The failure of immunocytochemical analysis to detect surface NGF

receptors on dermal fibroblasts may have been due simply to a lack of sensitivity. To determine if specific NGF binding sites are present on the surface of fibroblasts, we carried out ^{125}I-NGF equilibrium binding studies, using cultured Schwann cells as controls. Figure 3 shows that Schwann cells specifically bind ^{125}I-NGF over a large concentration range, as previously reported (DiStefano and Johnson, 1988a). However, fibroblasts showed no specific ^{125}I-NGF binding (Figure 3, inset). This result was verified by experiments in which ^{125}I-NGF was crosslinked to surface bound receptor using the heterobifunctional agent EDAC, followed by immunoprecipitation using MC192. Although this technique readily detected NGF receptors on Schwann cells (Figure 4), none were detected on fibroblasts.

Since intact NGF receptors were not detected on the surface of fibroblasts by binding methods or immunocytochemistry, we questioned whether these cells could be producing a soluble, perhaps secreted, form of the receptor protein. To test that idea, we added ^{125}I-NGF to medium conditioned by fibroblasts, crosslinked with EDAC, and immunoprecipitated the product with MC192, according to the procedures of DiStefano and Johnson (1988b). Figure 4 shows that no NGF binding activity was detectable in media conditioned by primary dermal fibroblasts, although under identical experimental conditions, abundant truncated NGF receptor could be detected within media conditioned by Schwann cells.

Detection of NGF receptors on fibroblasts by techniques that are based on ^{125}I-NGF binding could be confounded by the fact that fibroblasts produce NGF (Young et al, 1975; Bandtlow et al. 1986; Lindholm, 1988) which may bind to the receptor and reduce binding of exogenous radiolabelled ligand. To address this possibility, we labelled cell surface proteins directly with

^{125}I, and then immunoprecipitated cell homogenates with NGF receptor-specific antibodies. In addition to MC192, an anti-peptide antibody (p3) directed against a portion of the intracellular domain of the NGF receptor was used in these experiments. Figure 5 shows that although both MC192 and p3 effectively immunoprecipitate NGF receptor from Schwann cells surface labelled with ^{125}I (Panel A - 24 hour autoradiographic exposure), neither antibody recognized proteins in surface labelled dermal fibroblasts (Panel B - 6 week exposure).

PC12 cells respond to NGF treatment by transiently expressing the cellular proto-oncogene, c-fos (Millbrandt, 1986; Greenberg and Ziff, 1985) and production of fos mRNA in response to NGF treatment is good evidence of functional NGF receptors. Figure 6 shows that fos levels in NGF treated fibroblasts are no different from controls; under identical experimental conditions levels of fos mRNA are strongly induced within PC12 cells after 30 minutes incubation in media containing NGF. NGF-treated Schwann cells and C_6 glioma cells, other cell line that produce NGF (Murphy et al, 1977), also showed no NGF-induced increase in c-fos mRNA. Thus, of these cells, only PC12 cells contain receptors capable of transducing an intracellular signal in response to NGF, at least as measured by fos induction.

Fibroblasts Produce Normal, Unspliced NGF Receptor mRNA

One possible explanation for the lack of measurable NGF receptors on dermal fibroblasts is that the 3.7 kb NGF receptor mRNA expressed within these cells may be alternatively spliced and encode a different form of the receptor protein. This possibility was examined using the polymerase chain reaction (PCR). Southern blots of amplified NGF receptor cDNA sequences containing nucleotides 491 to 1526 revealed only a single NGF receptor species in

fibroblasts that was identical to that present in PC12 cells (1036 base pairs; Figure 7 - top and bottom panels). Although several cross-hybridizing cDNA species were identified on Southern blots washed to moderate stringency, cloning and sequencing of one of these (middle panel - arrow) showed that it did not arise from the NGF receptor gene (data not shown). Cloning and sequencing of the 500 base pair cDNA observed after high stringency washing revealed that it resulted from mispriming by primer BA8 beginning at nucleotide 1053 (Barker et al. submitted).

We also analyzed NGF receptor mRNA from fibroblasts directly using the RNase protection assay with labelled antisense RNA spanning sequences complementary to the cysteine-rich extracellular domain, the transmembrane domain and the intracellular domain. This was done as a confirmation of our PCR studies and also to rule out the possibility that forms of the message might exist that do not contain the sequences used for PCR priming. As shown in Figure 8 (lane E), only a single protected band was observed. Taken together, these PCR and RNase protection analyses suggest strongly that NGF receptor mRNA within fibroblasts is identical to the molecule cloned from PC12 cells (Radeke et al. 1986).

DISCUSSION

A large number of tissues express NGF receptors during development and in the adult but the role that the receptor plays outside the nervous system remains unclear. To begin to understand the physiological function of the receptor within nonneuronal tissues, it must first be characterized at the biochemical level. We chose to do this in dermal fibroblasts since dermal

mesenchyme expresses NGF receptor mRNA during development (Chesa et al. 1988; Byers et al. 1990; Heuer et al. 1990) and because cells within the perineurium, probably fibroblasts, appear to contain both NGF receptor mRNA and protein following tissue injury (Patil et al. 1990; Toma et al. submitted).

Our results show that cultured dermal fibroblasts contain authentic NGF receptor mRNA but we have been unable to detect NGF receptor protein. PCR and RNase protection analyses indicate that fibroblast NGF receptor mRNA is indistinguishable from that in NGF-responsive PC12 cells and in Schwann cells which do not respond to NGF. Therefore, absence of detectable protein is not due to cell-type specific alternative mRNA splicing. Also, fibroblasts transfected with NGF receptor cDNA express the protein on their cell surface indicating that fibroblasts have the ability to produce the protein (Radeke et al. 1986; Johnson et al. 1986; Welcher et al. 1990; Reddy et al. 1990).

We cannot rule out the possibility that NGF receptors are produced by cultured fibroblasts at levels that are undetectable by our methods. Certainly levels of receptor message are lower in cultured fibroblasts than in Schwann cells. If that is the case, then expression levels of the protein must also be extremely low since immunocytochemistry, equilibrium binding analysis, ^{125}I-NGF crosslinking, and direct surface labelling with ^{125}I failed to detect any protein with NGF binding activity or cross reactivity with NGF-receptor specific antibodies. Also, we did not detect any effect of NGF treatment on fibroblast levels of fos mRNA suggesting that fibroblasts, like Schwann cells, do not respond to NGF even though they contain mRNA coding for the receptor.

The absence of NGF receptor protein in the presence of NGF receptor mRNA has been reported for other systems. Undifferentiated neural crest cells

do not bind ^{125}I-NGF (Bernd, 1985) even though they clearly contain NGF receptor mRNA (Heuer et al. 1990) and developing trigeminal ganglia sensory neurons contain NGF receptor mRNA in the absence of measurable receptor (Davies et al. 1988; Wyman et al. 1990). One explanation for these results and also for our own is that NGF receptor mRNA produced within certain cells is not translated. Although we cannot rule this out, it would appear unlikely since NGF receptors become detectable on neural crest cell descendants and trigeminal sensory neurons as levels of their NGF receptor mRNA rise (Raivich and Kreutzberg, 1987; Ernfors et al. 1988; Davies et al. 1990). An alternative possibility is that NGF receptor protein is truncated to form soluble receptor and is thus not detected on cell surfaces (DiStefano and Johnson et al. 1988b). In this study, we were unable to detect the truncated form of the receptor in medium conditioned by fibroblasts although we know that the proteolytic activity that truncates the NGF receptor is present within fibroblasts (Barker et al. submitted). It is possible that low level production of the intact receptor combined with truncation due to proteolysis may prevent accumulation of the receptor at the cell surface. Although we were unable to detect intact or truncated NGF receptor by ^{125}I-NGF crosslinking in media conditioned by fibroblasts, we cannot rule out the possibility that receptor which is produced may already be bound to fibroblast-derived neurotrophins which include NGF (Young et al, 1976; Bandtlow et al. 1986; Lindholm, 1988) and brain derived neurotrophic factor (Acheson et al, submitted).

Mesenchymal cells (presumably fibroblasts) (Chesa et al. 1988; Heuer et al. 1990; Byers et al. 1990) and perineurial fibroblasts in rat sciatic nerve (Patil et al. 1990; Toma et al. submitted) produce levels of NGF receptor mRNA

and receptor protein that are easily detected by *in situ* hybridization and immunocytochemistry respectively. The failure of fibroblasts in culture to express detectable levels of the protein may mean that standard tissue culture medium does not provide some molecular constituent present *in vivo* which is necessary for expression of the protein. In that regard, FGF induces production of NGF receptor mRNA by MAH cells, a putative sympathoadrenal precursor, enabling them to respond to NGF treatment (Birren et al. 1990). Perhaps cytokines or growth regulating molecules regulate the production of NGF receptors in non-neuronal cells, and are necessary for their expression in cultured fibroblasts.

Why non-neuronal cells have mRNA coding for the NGF receptor or express the receptor in vivo is not clear. Fibroblasts and Schwann cells do not respond to NGF (as confirmed in this study). Schwann cells may indeed act as a substrate in providing NGF to regenerating nerve cells (Taniuchi et al. 1986), but it is unlikely that fibroblasts have a similar function physiologically, since they are not in direct contact with nerve processes. Perhaps these receptors have other functions. They may bind and respond to other members of the NGF family of proteins or they may mediate effects of these molecules which have not been revealed by currently available bioassays. The widespread appearance of NGF receptor protein and mRNA in non-neuronal cells suggest strongly, however, that this molecule and its ligand may have important roles in tissue function that are not related to the growth and survival of neurons.

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Figures

Fig. 1 Detection of NGF receptor mRNA in primary Schwann cells and dermal fibroblasts. 10 ug of total RNA was electrophoresed through 1% agarose, transferred to nitrocellulose, hybridized with an antisense cRNA encoding nucleotides 700 to 1245 and washed as described in Materials and Methods. Blots were exposed to Kodak XAR film at -70° C with an intensifying screen for 12 hours (Schwann cell RNA) or 4 days (fibroblast RNA).

NGF RECEPTOR mRNA IS PRESENT IN FIBROBLASTS

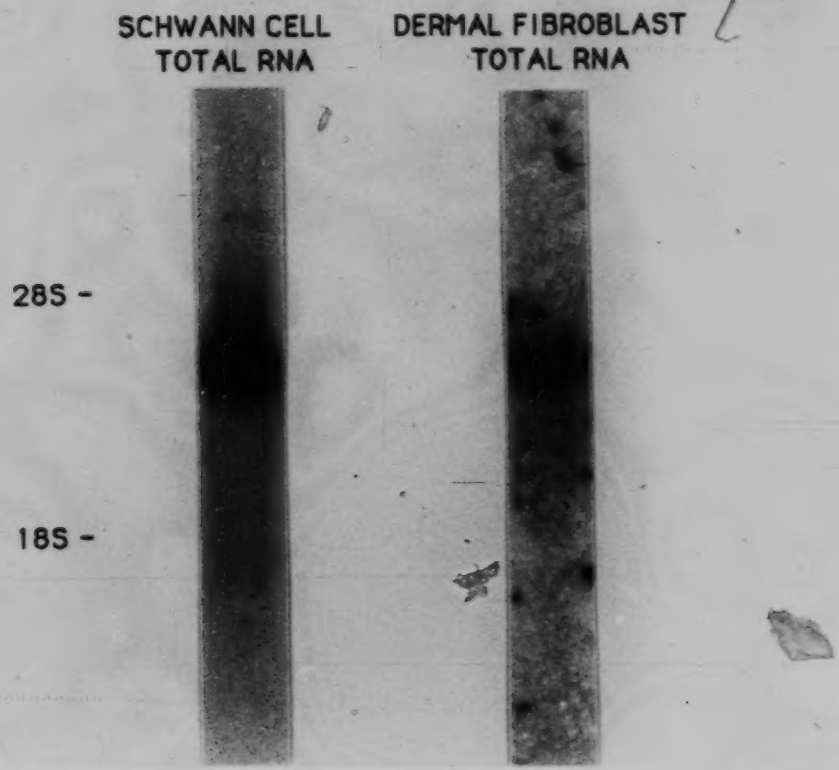
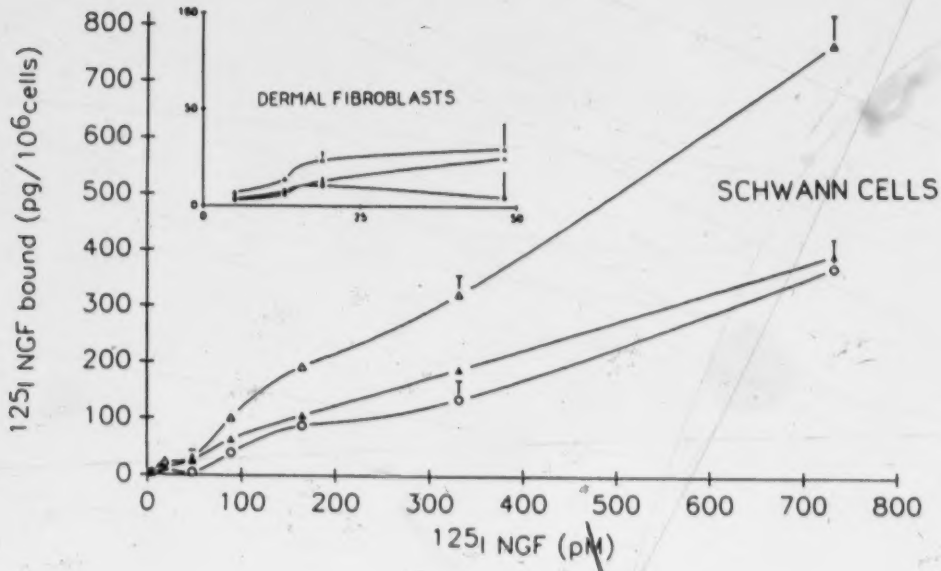


Fig. 2 **MC192 immunoreactive material in dermal fibroblasts.** Permeabilized primary dermal fibroblasts were stained with MC192, a rat NGF receptor specific mouse monoclonal antibody (Panels A (63X) and B (40X)), or with MOPC21, a control IgG_1 mouse monoclonal antibody (Panel C (40X)) as described in Materials and Methods. In some cells within some cultures, punctate staining was observed throughout the cytoplasm in cells treated with MC192 that was not observed in control cultures; however, it should be emphasized that this result was highly variable and positive staining did not occur in all cultures (see text).

Fig. 3 **Equilibrium binding of ^{125}I-NGF to dermal fibroblasts did not detect surface NGF binding sites.** Equilibrium binding of ^{125}I-NGF to Schwann cell and dermal fibroblast surfaces was performed as described in Materials and Methods. Empty triangles indicate total binding, filled triangles indicate nonspecific binding determined in the presence of 0.4 uM unlabelled NGF and open circles indicate specific binding. Standard deviations are shown by error bars on the curves for total and specific binding. Where error bars do not appear, the standard deviation is incorporated into the sizes of the symbol.

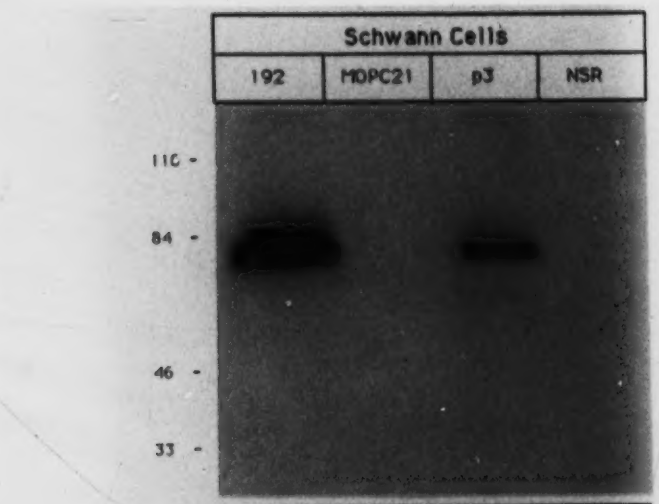
Fig 4. **^{125}I-NGF crosslinking did not detect NGF receptors on dermal fibroblast surfaces or within conditioned media.** Schwann cells, dermal fibroblasts or media conditioned by these cells were incubated with ^{125}I-NGF, crosslinked with EDAC, immunoprecipitated with MC192 and analyzed by SDS-PAGE followed by fluorography. For cold competitions, samples were incubated with ^{125}I-NGF in the presence of a 250-fold excess of unlabelled NGF.

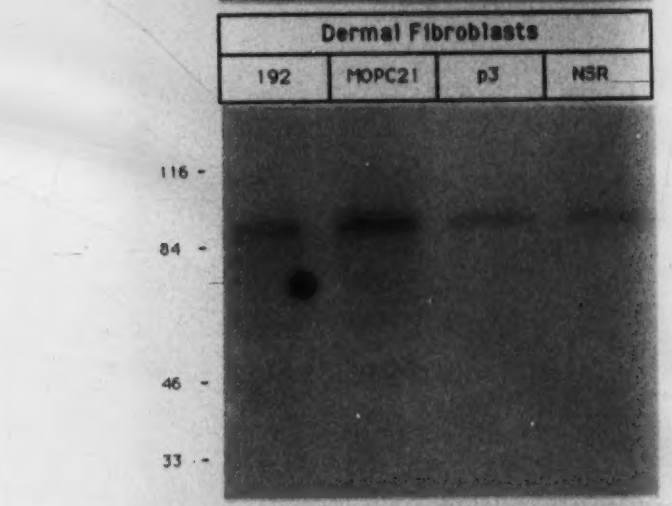


EDAC MEDIATED 125I-NGF CROSSLINKING

Fig. 5 **NGF receptor is detected by direct cell surface labelling in Schwann cells but not in dermal fibroblasts.** Cell surface proteins on Schwann cells (Panel A) and fibroblasts (Panel B) were labelled with ^{125}I using lactoperoxidase, solubilized and immunoprecipitated using either MC192, MOPC21, p3 or non specific rabbit IgG and analyzed by SDS-PAGE followed by fluorography. The autoradiograph shown in Panel A was exposed for 24 hours; that shown in Panel B for 6 weeks.

Fig. 6 **Fos mRNA is not induced in fibroblasts following NGF exposure.** 10 ug of total RNA was electrophoresed through 1% agarose, transferred to nitrocellulose, hybridized with labelled cDNA prepared by random priming of rat fos cDNA encoding the entire fos open reading frame and washed as described in Materials and Methods. Blots were exposed to Kodak XAR film at -70° C with an intensifying screen for 3 hours.


Schwann Cells
192
MOPC21
p3
NSR
110 -
84 -
46 -
33 -


Dermal Fibroblasts
192
MOPC21
p3
NSR
116 -
84 -
46 -
33 -

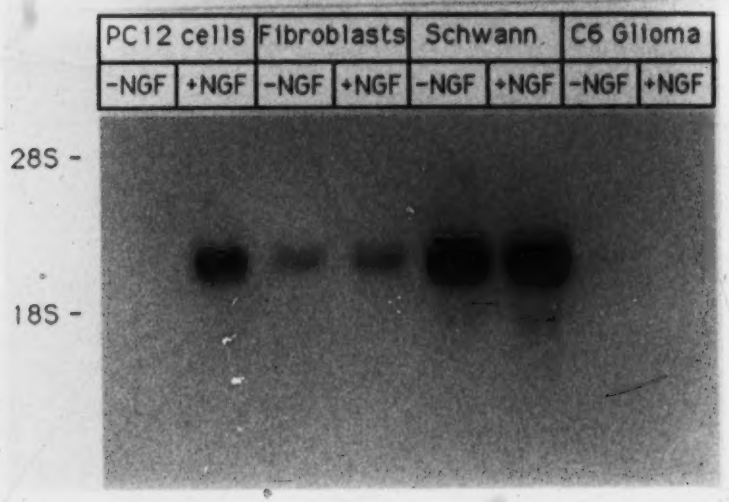

PC12 cells
Fibroblasts
Schwann
C6 Glioma
-NGF
+NGF
-NGF
+NGF
-NGF
+NGF
-NGF
+NGF
28S -
18S -

Fig. 7 **PCR analysis of PC12 cell and dermal fibroblast NGF receptor mRNA.** NGFR cDNA between nt 491 to 1526 were amplified from PC12 and dermal fibroblast cDNA using PCR as described in Materials and Methods. The region of the NGF receptor mRNA analyzed is shown schematically (Panel A). All PCR products were subjected to electrophoresis on 1% TBE gels, transferred to nitrocellulose, and analyzed by Southern blotting using [^{32}]P-labelled cRNA encoding nt 700 to 1245 of the NGF receptor sequence (Panel B). For moderate stringency, blots were washed at 55° in 0.1X SSC; for high stringency; blots were washed in 0.05X SSC at 66° C.

PC12 and Dermal Fibroblast NGF Receptor PCR Products on Southern Blots

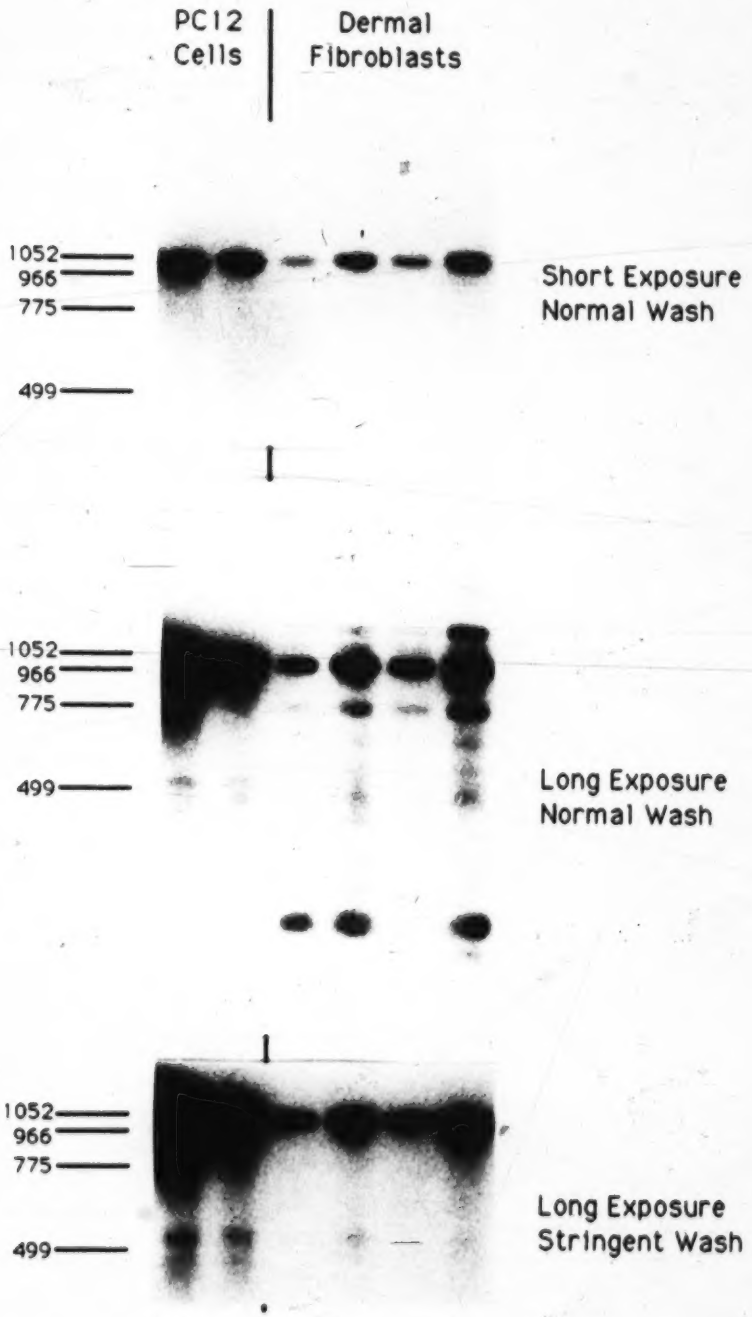


Fig. 8 **RNase protection analysis of NGF receptor mRNA.** A [^{32}P]-labelled cRNA complementary to nt 700 to 1245 of the rat NGF receptor mRNA was used in RNase protection assays as described in the Materials and Methods. Assays were performed on 10 ug total RNA isolated from neonatal skin (lane A), adult skin (lane B), and adult wounded skin (lane C) which are rich in fibroblasts and contain significant amounts of NGF receptor mRNA. Also analyzed was NGF receptor mRNA (2 ug, lane D and 10 ug, lane E) in total RNA isolated from dermal fibroblasts and on 20 ug yeast tRNA (lane F). Lane G contains intact undigested cRNA.

A
B
C
D
E
F
G

CONCLUSIONS AND CLOSING REMARKS

The Type II NGF receptor exists either as a surface bound transmembrane molecule or as a soluble protein probably with only an extracellular portion. Both of these proteins bind NGF; the surface bound form of the receptor appears to be required for transduction of the NGF signal but the role of the soluble receptor remains unknown. A major goal of my thesis was to determine which molecular mechanisms account for the production of the soluble receptor. Several lines of evidence are consistent with the hypothesis that the soluble form is formed post-translationally from intact NGF receptor in primary Schwann cells. First, by PCR and RNase protection analysis, we found no evidence for alternative NGF receptor mRNA species which could account for the soluble form. Second, although present in conditioned media, the soluble form of the receptor was not detected intracellularly. Third, pulse-chase analysis indicated that the truncated receptor was produced slowly, well after surface NGF receptor was fully processed. This slow rate of accumulation of the truncated receptor in conditioned media is not consistent with the hypothesis that this protein is synthesized and secreted directly but is consistent with the hypothesis that the truncated receptor is formed post-translationally from the intact membrane bound form of the molecule. Finally, fibroblasts transfected with the Type II NGF receptor cDNA, which cannot be alternatively spliced, produce both surface bound and soluble receptor. Together, this experimental evidence strongly suggests that the truncated form of the receptor is formed post-translationally from intact receptor.

Of the primary non-neuronal cell types which express the NGF receptor, Schwann cells are by far the best characterized. To begin to analyze NGF

receptor biochemically on other non-neuronal cell types, we chose to study dermal fibroblasts as a model system since tissues rich in fibroblasts express NGF receptor during development and cells within perineurium, probably fibroblasts, express NGF receptor mRNA and protein within 3 hours of tissue injury. Our studies show that although NGF receptor mRNA which was indistinguishable from that within PC12 cells was clearly detected within fibroblasts, no evidence of NGF receptor protein was obtained by several sensitive techniques. The most obvious explanation for this is that the receptor is present at levels undetectable by our techniques. However, other explanations must be considered. One is that the NGF receptor mRNA within fibroblasts might be untranslated when present at low levels. However, this is unlikely since fibroblasts transfected with NGF receptor cDNA produce abundant NGF receptor protein and within perineurial cells, mainly fibroblasts, NGF receptor protein is clearly detectable after wounding. Another possibility is that the low levels of NGF receptor produced within these cells does not accumulate at the membrane because it is truncated post-translationally to produce soluble NGF receptor. Although we were unable to detect the truncated form of the receptor in media conditioned by dermal fibroblasts, we cannot rule out the possibility that truncated receptor may be undetectable because it is bound to endogenous neurotrophins and thus unavailable to bind radiolabelled NGF. Therefore, the nature of the NGF receptor protein produced by these cells remains unresolved.

It is becoming increasingly clear that neurotrophins and their receptors represent an extraordinarily complicated biological system. The Type II NGF receptor exists as a transductory complex on responsive neurons and as a soluble binding protein. Because it binds not only NGF but also BDNF, it may

also be a component of more than one neurotrophin receptor and likely acts as a soluble binding protein for neurotrophins other than NGF as well. Cells that bear NGF receptors but do not respond to NGF may contain NGF receptors for roles other than signal transduction but the possibility that NGF receptors on these cells may transduce the signal of other neurotrophins must also be considered. The roles of the soluble receptor remain completely uncharacterized but may be significant. Unravelling the biology of such a complex system has been, and will continue to be, an exciting task.